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Article

Keywords: GTPase, adherens junction, polarity, RAS, scribble, Afadin, PDZ domain, FHA 50 domain, RAP

Posted Date: November 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1074956/v1>

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Version of Record: A version of this preprint was published at Nature Communications on August 5th, 2022. See the published version at <https://doi.org/10.1038/s41467-022-32335-8>.

Afadin couples RAS GTPases to the polarity rheostat

Scribble

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Summary

AFDN/Afadin is required for establishment and maintenance of cell-cell contacts and is a unique effector of RAS GTPases. The biological consequences of RAS signalling to AFDN are unknown. Here, we use proximity-based proteomics to generate an interaction map for the long and short isoforms of AFDN, identifying the polarity protein SCRIB/Scribble as the top hit. We reveal that the first PDZ domain of SCRIB and the AFDN FHA domain mediate a direct but non-canonical interaction between these important adhesion and polarity proteins. Further, the dual RA domains of AFDN have broad specificity for RAS and RAP GTPases, and KRAS co-localizes with and promotes AFDN-SCRIB complex formation. Knockout of *AFDN* or *SCRIB* in MCF7 epithelial cells disrupts MAPK and PI3K activation and inhibits cell motility in a growth factor-dependent manner. These data have important implications for understanding why cells with activated RAS have reduced cell contacts and polarity defects, and finally begin to characterize AFDN as a RAS effector.

Keywords: GTPase; adherens junction; polarity; RAS; scribble; Afadin; PDZ domain; FHA domain; RAP

Introduction

RAS small GTPases function as molecular switches by undergoing nucleotide-dependent conformational exchange. When GTP-bound, RAS GTPases are bound directly by effector proteins that are responsible for transmitting signals to diverse cellular pathways¹⁻⁴. This is the accepted paradigm of GTPase signalling, yet most of our understanding derives from study of a small subset of GTPase-effector partners and the biological significance of many proposed RAS effectors remains uncertain (Figure 1A).

The RAS subfamily of small GTPases consists of 35 members and includes the archetypal oncoproteins H-, K- and N-RAS, along with related GTPases of the RAP, RAL, RIT and RHEB families⁵. Effectors bind activated RAS GTPases through RAS-Association domains (RAs) or RAS Binding Domains (RBDs). Structural biology studies have revealed that RBD/RA domains share a common ubiquitin fold and interact with the same epitope on RAS⁶⁻¹⁰. There are over 50 predicted RBD/RA domains in the human proteome present in an array of proteins with diverse associated domains¹¹. While the RAF and PI3K effectors have received the most attention due to their role in proliferation and survival, there are numerous effectors that are highly conserved through evolution that bind activated RAS *in vitro* and *in vivo*. These include the RALGEF proteins^{12,13}, the RASSF death effectors¹⁴⁻¹⁷ and the adhesion protein AFDN (also Afadin/AF6/MLLT4).

AFDN, an ortholog of *Drosophila* canoe, is a unique RAS effector with two N-terminal RA domains that plays an essential role in the formation and maintenance of adherens junctions (AJs)¹⁸⁻²⁰. Originally identified as a fusion partner of the MLL histone methyltransferase in leukemia²¹, AFDN was later shown to directly bind activated HRAS, RAP1A and RAP2A^{1,22}. Further evidence links AFDN to R-RAS and M-RAS²³, revealing potentially plastic GTPase-effector signalling. In *Drosophila*, canoe regulates the connection between AJs and actin and maintains polarity during apical constriction in a RAP-dependent manner^{19,20}. A crystal structure of the first RA domain (RA1) of AFDN bound to HRAS validated its capacity to associate with RAS *via* a ubiquitin-like domain¹¹, but a biological function for this interaction has not been explored. Intriguingly, recent application of proximity-based proteomics (BioID) has consistently found the most proximal effector of activated RAS in cells is not RAF or PI3K, but AFDN^{24,25}. While RAS binding to AFDN may not directly stimulate proliferation, the metastatic nature of RAS mutant cancers makes study of this GTPase-effector complex a high priority.

Signal transduction from activated GTPases through effector proteins is dependent on their uniquely associated protein domains. For AFDN (Figure 1B), this includes a forkhead-associated (FHA) domain with no known binding partners, a dilute (DIL) domain that binds ADIP to influence actin cytoskeletal organization at AJs²⁶, and a PSD95/DLG1/ZO-1 (PDZ) domain with numerous binding partners involved in the cell adhesion apparatus (Nectins²⁷, JAM-A²⁸, and Eph receptors²⁹). Other partners include the tight junction protein ZO-1³⁰, the AJ cytoskeletal regulator PLEKHA7³¹ and the AJ components p120-catenin and α -catenin^{30–32}. The C-terminal helical region of AFDN binds to actin, deemed critical to its role in regulating cell adhesion. This region is absent from a truncated isoform called s-AFDN (iso2) compared with the longer l-AFDN (iso1)^{33,34}. A complete signalling network for AFDN has yet to be defined and the impact of GTPase binding on its interactors is unknown. Despite this, AFDN plays a key role in regulating cell adhesion in metazoans and signalling from RAS subfamily GTPases to AFDN could have important implications for metastasis in RAS-driven cancers. Indeed, AFDN mutations were identified as drivers in breast cancer³⁵, and loss of AFDN from cell-cell contacts induces migration and invasion in endometrial³⁶, pancreatic³⁷ and breast cancers³⁸ as well as glioblastomas³⁹.

Here, we use proximity labelling coupled to mass spectrometry to build a comprehensive protein interaction network for the RAS effector AFDN. Our data corroborate known partners and uncover many novel interactions, most notably direct binding to the tumour suppressor polarity protein SCRIB (ortholog of *Drosophila* scribbled). We provide evidence that AFDN and SCRIB associate in cells *via* a non-canonical FHA-PDZ domain interaction, and that activated RAS GTPases promote formation of this complex. Loss of *AFDN* or *SCRIB* impacts RAS activation of the MAPK and PI3K pathways and disrupts motility in a growth factor-dependent manner. These results have important implications to both RAS-induced tumourigenesis and the normal development and maintenance of cell-cell contacts in epithelial layers.

Results

The AFDN Interaction Network

To understand the implications of AFDN as a GTPase effector we required a comprehensive map of the AFDN interaction network. To address this we used proximity biotinylation coupled to proteomics (BioID)⁴⁰. For bait, we generated stable HeLa cell lines that

express either the long (iso1) or short (iso2) isoforms of AFDN fused with biotin ligase (BirA*) and the FLAG epitope in a Tet-inducible manner (Figure S1A). Data from two biological replicates were filtered with SAINT⁴¹, revealing a total of 95 high-confidence bait-prey relationships (Table S1). Gene Ontology (GO) analysis of the full set of interactors provides insight to the localization and function of AFDN (Figure 1C). Both AFDN iso1 and iso2 associate primarily with signalling, adhesion, transport and morphogenesis proteins at the plasma membrane, with evidence that either AFDN or its prey proteins shuttle to the endoplasmic reticulum (ER), Golgi and mitochondria. 46/95 (48%) of identified proteins were found using both AFDN iso1 and iso2 as baits, including the majority of those with the highest spectral counts. The top 25 coincident hits are listed in Figure 1D. These proteins have a variety of associated domains but are particularly enriched in PDZ domains, typical of proteins involved in cell adhesion. As the majority of small GTPases *in vivo* are in the inactive, GDP-bound conformation we did not expect detection of transient GTPase partners using this approach, however, it is notable that several other RA domain proteins (and putative RAS effectors) are present in the dataset (TP53BP2/ASPP2, RASSF8, and PPP1R13B/ASPP1^{17,42}). Several hits were isoform-specific and are listed in Figure S1B-C. These were primarily ER/Golgi proteins for iso1 of AFDN, and plasma membrane proteins for the shorter iso2 that is deficient in actin binding. There were several known AFDN partners identified that corroborate the efficacy of this approach, including cadherin-associated CTNND1/p120-catenin and CTNNA1/ α -catenin, and the cell adhesion receptors PVRL2/nelectin-2 and PVRL3/nelectin-3. The most prominent preys identified using either bait, however, had not previously been associated with AFDN function: the master polarity protein Scribble (SCRIB) and the adhesion protein Discs Large Homolog 5 (DLG5). While association with all identified preys may have important implications for AFDN function, there are intriguing data linking SCRIB to RAS GTPase-induced metastasis⁴³⁻⁴⁶ and regulation of AJs. Loss of *AFDN* and *SCRIB* synergistically promotes RAS-induced cell growth⁴⁷ and invasion⁴⁸, and canoe and scribbled are co-determinants of AJ formation in *Drosophila* that co-localize at apical contacts in epithelial layers⁴⁹. We therefore chose to investigate a possible direct link between AFDN and SCRIB.

Protein Domain Mapping the AFDN-SCRIB Interaction

To corroborate the BioID result we first examined AFDN and SCRIB subcellular

localization. In human epithelial HeLa cells, used for the proteomic analysis, we observed overexpressed AFDN and SCRIB co-localize around the cell cortex and in the cytoplasm (Figure 2A). As these proteins are typically studied in cells that establish AJs and apical-basal polarity, we immunostained endogenous AFDN and SCRIB in the mammary epithelial MCF7 cell line. Though SCRIB is generally considered a basolateral marker and AFDN more apical, z-stack projections revealed significant overlap in their position at cell-cell contacts (Figure 2B). To test if these proteins complex, we co-expressed full-length SCRIB and AFDN with N-terminal epitope tags. Upon immunoprecipitation (IP) of AFDN we observed significant and specific co-precipitation of SCRIB (Figure 2C). We could also detect endogenous SCRIB in an IP of endogenous AFDN (Figure 2D). These data corroborate a direct AFDN-SCRIB complex.

As both proteins comprise several distinct domains, we sought to map this interaction to specific regions. First, bacterial expression constructs encoding N-terminal glutathione S-transferase (GST) fusions of defined fragments covering the length of AFDN were generated (Figure 2E). Following their expression and purification from *E. coli*, the fragments were mixed with glutathione beads and lysates of mammalian cells expressing potential interaction partners. Figure 2F shows that both overexpressed SCRIB and endogenous SCRIB were precipitated specifically by a fragment encompassing the AFDN FHA domain. It also verified that the RA1-RA2 region binds activated KRAS (G12V, constitutive GTP-bound mutant) but not wild-type KRAS (predominantly GDP-bound *in vivo*). Mixing with lysates expressing either N- or C-terminal halves of SCRIB revealed an interaction with only the N-terminal region, comprising the leucine-rich repeats (LRRs) and the first of four PDZ domains (PDZ1). To resolve which region of SCRIB binds AFDN we generated GST-fusions of the SCRIB LRR and PDZ1 domains (Figure 2G). These proteins were purified and mixed with lysates from cells expressing either AFDN iso1 or iso2. Figure 2H demonstrates that the PDZ1 domain of SCRIB, and not the LRR region, specifically precipitated iso1, iso2 and also endogenous AFDN. These data suggest that the FHA domain of AFDN and the PDZ1 domain of SCRIB mediate an interaction between the two proteins. To confirm this, we generated domain deletion constructs to express either AFDN Δ FHA (missing residues 370-600) or SCRIB Δ PDZ1 (missing residues 718-820) in context of the full-length proteins. Compared to wild-type, AFDN Δ FHA did not co-precipitate with SCRIB (Figure 2I) and SCRIB Δ PDZ1 no longer bound to AFDN (Figure 2J). These data corroborate an FHA-PDZ mediated interaction and propose SCRIB as the first binding partner of

the AFDN FHA domain.

A Phospho-Independent FHA-PDZ1 Complex

We sought to determine the molecular mechanism of this interaction and whether these protein modules associate directly, aided by an available NMR structure of the AFDN FHA domain (PDBid 1WLN, unpublished) and two structures of SCRIB PDZ1 (PDBids 5VWK⁵⁰ and 1X5Q, unpublished). Archetypal PDZ domain binding motifs are found at the C-termini of proteins, though some PDZ interactions occur at atypical binding sites^{51–53}. SCRIB PDZ1 precipitated both AFDN iso1 and iso2, suggesting the interaction does not involve the AFDN C-terminus. To exclude the possibility that our bacterially expressed, GST-FHA domain was itself providing a PDZ binding motif we generated variants with distinct C-termini. AFDN FHA domain constructs ending at residues 590 (-ENR), 599 (-PEL) or 610 (-RES) were equally efficient at precipitating SCRIB from cell lysates (Figure 3A). This suggested the presence of a non-canonical, internal PDZ binding site in the AFDN FHA domain.

FHA domains are protein-interaction modules classically involved in binding to phosphothreonine (pTHR) motifs^{54,55}. It was conceivable that a pTHR site exists in the SCRIB PDZ1 domain that serves as a binding site for AFDN. Sequence and structural alignments revealed, however, that the AFDN FHA domain does not include two positively charged residues critical for coordinating pTHR interactions in other FHA domains (Figure 3B and S2A). To confirm this experimentally, we repeated the co-IP of full-length AFDN and SCRIB following incubation of the lysates with phosphatase (Figure 3C). As predicted, treatment of the lysates with phosphatase did not disrupt AFDN-SCRIB complex. We also performed a complete phospho-proteomic analysis of SCRIB to identify potential phosphorylation sites in PDZ1 (Figure S2B). Several sites were mapped, but no SER, THR or TYR residues in the PDZ1 domain were found phosphorylated. We also tested whether the alternatively spliced exon 9 (ex9) of *AFDN*, which results in removal of residues 393–407 in a loop region of the FHA domain, would alter its complex with SCRIB. IP of AFDN Δ ex9 following its co-transfection with SCRIB demonstrated that ex9 encoded residues do not affect SCRIB binding (Figure S3A). Together, these data indicate that the AFDN-SCRIB interaction is phospho-independent with no significant role for the alternatively spliced ex9 residues, and their complex could be studied *in vitro* with purified proteins.

The SCRIB PDZ1 domain proved well folded and soluble using previously defined boundaries (residues 718-820). For the AFDN FHA domain, we generated five GST fusion constructs based on the available structure and on secondary structure predictions that revealed a helical region spanning residues 563-576 (Figure S3B). We mixed purified SCRIB PDZ1 domain with each of the recombinant FHA domains, and results indicated that the predicted α -helix is essential for a direct interaction (Figure 3D). The core FHA domain as determined in the NMR structure (381-501) did not bind PDZ1. The importance of the C-terminal extended region was corroborated by NMR spectroscopy. $^1\text{H}/^{15}\text{N}$ -HSQCs of ^{15}N -labelled AFDN FHA revealed the FHA domain fold is conserved in the extended protein, and chemical shifts specific to the C-terminal helix were identical to those demonstrating line broadening upon titration of unlabelled SCRIB PDZ1 (Figure 3E). Despite this, a construct encompassing only AFDN residues 531-580 did not bind SCRIB (Figure 3F), indicating this helical extension and the core FHA domain are both required to bind PDZ1. This α -helix bears significant homology to a C-terminal helix in the Rad53 FHA domain⁵⁶, which stabilizes the FHA fold. Thus, we resolved a direct binding module comprising the SCRIB PDZ1 (718-820) and a C-terminally extended FHA domain of AFDN (371-580).

To measure the affinity of purified FHA and PDZ1 domains we used isothermal titration calorimetry (ITC). The protein domains bound with an equilibrium dissociation constant (K_d) of 14.8 μM in 150 mM NaCl (Figure 3G). This affinity is comparable to previously studied PDZ domain interactions^{50,53,57,58}. We also assessed whether deletion of ex9 residues in the FHA domain would alter affinity. Purified FHA domain Δex9 bound PDZ1 with a K_d of 22.9 μM , slightly weaker than the FHA domain with ex9 (Figure S3C). Finally, prototypical PDZ domain interactions are predominantly driven by insertion of a hydrophobic residue into a deep hydrophobic pocket of the PDZ domain. To establish if there are electrostatic contributions to the FHA-PDZ1 complex we performed ITC at a lower salt concentration (20 mM NaCl; Figures S3C/D) and observed significantly improved affinity (K_d of 5.7 μM). To map the interaction site on the SCRIB PDZ1 domain we used NMR spectroscopy. BMRBID 11207 was used to assign 71% of the peaks in a $^1\text{H}/^{15}\text{N}$ -HSQC of ^{15}N -labelled SCRIB PDZ1. Titration of unlabelled AFDN FHA domain resulted in extensive broadening for the majority of PDZ1 peaks, and significant chemical shift perturbations for some (Figure S3E). Figure 3H reports intensity ratios for all assigned peaks (unbound PDZ1 versus FHA domain-bound) and was used to map the

binding interface. Using the structure of SCRIB PDZ1 bound to a C-terminal peptide from β -PIX (PDBid 5VWK) and our NMR data, we resolved that the AFDN FHA domain binds SCRIB PDZ1 in the classic C-terminal motif pocket (Figure 3I). We have therefore elucidated a non-canonical FHA-PDZ domain interaction that is phospho- and C-termini-independent involving both electrostatic interactions and the archetypal hydrophobic PDZ domain binding pocket.

RAS GTPases complex with AFDN-SCRIB

AFDN has been shown to bind several RAS subfamily GTPases and is commonly described as a RAS/RAP effector. The dual N-terminal RA domains of AFDN are a unique and evolutionarily conserved feature. To determine whether activated GTPases could complex with AFDN and SCRIB we first considered KRAS. IP of AFDN following its co-expression with an activated mutant of KRAS (G12V) established both KRAS and endogenous SCRIB were co-precipitated with AFDN (Figure 4A). A comparable level of SCRIB was detected with AFDN in the absence of activated KRAS, suggesting a ternary KRAS-AFDN-SCRIB complex. Similar results were obtained with endogenous AFDN when KRAS-G12V was co-expressed with FLAG-SCRIB (Figure 4B). We then screened whether AFDN could interact with several related RAS and RAP family GTPases. We purified the dual AFDN RA1-2 domains (residues 6-370) as a GST-tagged protein, as well as a GST-RBD from the archetypal RAS effector BRAF. We expressed 8 distinct RAS subfamily GTPases with activating mutations (constitutively GTP-bound, based on RAS G12V) in mammalian cells and mixed lysates with either GST-AFDN RA1-2 or GST-BRAF RBD (Figure 4C). BRAF specifically bound the H-, K- and N-RAS GTPases, while AFDN demonstrated a broadened specificity. In particular, the RAP GTPases RAP1B and RAP2C bound AFDN with similar intensity to H-, K- and N-RAS, and RAP1A to a lesser extent. RAP2B and the distantly related DIRAS2 showed very weak or no interaction. Of note, the dual RA domains of AFDN precipitated activated RAS with similar efficiency to BRAF despite a lower affinity of the individual RA1 domain¹¹. We also confirmed that full-length AFDN can associate with activated RAP1B, RAP2C and KRAS (Figure S4A). These data indicate that AFDN binds multiple GTPases in the RAS subfamily and that SCRIB is maintained in an AFDN-KRAS complex.

How GTPases alter AFDN interactions is unknown. As AFDN and SCRIB may regulate adhesion and polarity downstream of RAS, we sought to examine how RAP or KRAS binding

affects AFDN binding to SCRIB. We co-transfected full-length AFDN and SCRIB with KRAS-G12V, RAP1B-G12V or RAP2C-G12V and used wild-type KRAS or no GTPase as controls. We consistently observed increased SCRIB co-precipitated with AFDN in the presence of KRAS-G12V compared to RAP GTPases or controls (Figure 4D). Densitometry analysis of blots from five separate experiments indicated an 8-fold increase in SCRIB co-precipitation with AFDN when KRAS-G12V was co-expressed (compared with wild-type KRAS). A SCRIB protein that can not bind AFDN (SCRIB Δ PDZ1) did not alter AFDN association with KRAS-G12V and was not co-precipitated (Figure 4E). These data suggest that activated KRAS does not disrupt, and ostensibly augments, an AFDN-SCRIB complex.

We next considered whether AFDN, KRAS and SCRIB are co-localized in cells. All three proteins are typically located at the plasma membrane, so we chose to examine whether expression of oncogenic KRAS might disrupt the subcellular localization of AFDN and SCRIB in MCF7 cells, which are wild-type for *NRAS*, *KRAS* and *HRAS*. We first established that expression of GFP alone or GFP-tagged, wild-type KRAS does not significantly alter AFDN or SCRIB localization and does not co-localize with these proteins (Figure 4F and S4B). In fact, wild-type KRAS was observed ubiquitously across the plasma membrane, including along the basolateral surface. In contrast, expression of KRAS-G12V markedly disrupted cell-cell contacts and was noticeably co-localized with endogenous AFDN and SCRIB, as determined by z-plane projections (Figure 4G). Most cells expressing KRAS-G12V extract from the monolayer and were observed on the apical surface or underneath cells of the monolayer. In these cells, we could no longer detect AFDN or SCRIB at the membrane, suggesting their interaction with KRAS is dependent on intact cell-cell contacts. Thus, activated KRAS GTPases are primed for AFDN and SCRIB complex at epithelial cell junctions and these cells ultimately detach.

CRISPR KO of AFDN or SCRIB disrupts adhesion and polarity

To understand how loss of AFDN and/or SCRIB impacts RAS localization and further explore the KRAS-AFDN-SCRIB complex we generated CRISPR knockouts (KOs) of *AFDN*, *SCRIB* or both *AFDN* and *SCRIB* together in MCF7 cells (Figure 5A). KO of *AFDN* produced a marked mesenchymal phenotype, with elongated and flattened cells lacking discernible cell-cell contacts, while *SCRIB* KO cells appeared to retain cell-cell adhesion but were smaller, rounder and grew in multicell layers (Figures S5A/B). We first sought to validate our elucidated interactions, whereby AFDN scaffolds a complex between SCRIB and RAS GTPases (Figure

5B). This implies that KRAS would no longer co-precipitate with SCRIB in cells lacking AFDN. To test this, we overexpressed activated KRAS-G12V and SCRIB in the presence or absence of AFDN in *AFDN* KO cells, and immunoprecipitated SCRIB. Figure 5C shows KRAS only co-precipitated with SCRIB when AFDN was re-expressed, supporting our model. We next examined the localization of AFDN, SCRIB and KRAS in the KO cell lines. SCRIB localization was completely disrupted in *AFDN* KO cells, with endogenous SCRIB found dispersed throughout the cytoplasm (Figure 5D). Expression of GFP-tagged KRAS-G12V in these cells did not alter SCRIB localization, and KRAS itself was localized at the plasma membrane. Wild-type KRAS displayed a similar localization (Figure S5C). In *SCRIB* KO cells, AFDN was retained at sites of cell-cell contact (Figure 5E). z-plane projections of these regions could not distinguish whether the apical-basal distribution of AFDN is altered, as *SCRIB* KO cells do not form an organized monolayer but rather grow in layers 1-3 cells thick. We could not find *SCRIB* KO cells expressing GFP-KRAS-G12V that remained within the monolayer. Disengaged cells were observed on the apical or basal surfaces of cells not expressing KRAS. As with the parental MCF7 line (Figure 4G), endogenous AFDN did not appear at cell contacts in KRAS-G12V expressing cells that detached from the monolayer. In contrast, cells expressing wild-type KRAS were retained in the monolayer and AFDN remained at cell contacts (Figure S5D). Together, these results corroborate a KRAS-AFDN-SCRIB complex and suggest that KRAS is membrane localized and presumably functional in cells lacking AFDN or SCRIB.

To study the AFDN-SCRIB complex we generated rescue lines expressing either full length AFDN or AFDN Δ FHA in *AFDN* KO cells, and full length SCRIB or SCRIB Δ PDZ1 in *SCRIB* KO cells. Lentiviruses encoding these proteins with N-terminal GFP tags were transduced (or GFP alone control), and stable cell lines generated by selection (Figure S5E). We first examined whether re-expression of AFDN would restore SCRIB localization at the cell cortex of *AFDN* KO cells. Indeed, expression of either wild-type or Δ FHA AFDN induced SCRIB membrane localization and re-established cell-cell contacts, while SCRIB remained cytoplasmic in the GFP alone control (Figure S6A). AFDN was retained at sites of cell contact in *SCRIB* KO cells, and this did not change with re-expression of SCRIB or SCRIB Δ PDZ1 (Figure S6B). A closer examination of z-plane projections revealed that both AFDN Δ FHA and SCRIB Δ PDZ1, though enriched at the cell cortex, were also present more generally in the cytoplasm and did not completely overlap with endogenous SCRIB or AFDN, respectively

(Figure 6A/B). This can be directly observed when we consider projections of 10 μ m length along cell-cell contacts, for AFDN rescues in Figure 6C and for SCRIB rescues in Figure 6D. These data show in multiple independent cells that wild-type AFDN and SCRIB localization is overlapping. Conversely, AFDN Δ FHA remained apical to endogenous SCRIB in *AFDN* KO cells, and SCRIB Δ PDZ1 basal to endogenous AFDN in *SCRIB* KO cells. This supports a model whereby a direct FHA-PDZ1 interaction is essential for proper localization of AFDN and SCRIB at cell contacts with apical-basal polarity.

Loss of AFDN or SCRIB alters RAS signalling and cell motility in response to EGF

Finally, we explored whether loss of *AFDN* or *SCRIB* would alter signalling downstream of RAS. Stimulation of starved cells by growth factor (EGF) was used to promote activation of endogenous RAS. We first assessed whether the two major RAS proliferation and survival pathways, MAPK and PI3K, were activated and whether the kinetics of this activation were altered. Time courses of EGF stimulation in the parental MCF7 line, the *AFDN* or *SCRIB* KO lines were performed and pathway activation measured by induction of pERK (MAPK) or pAKT (PI3K) (Figures 7A-C). Quantification of pERK (Figure 7D) and pAKT (Figure 7E) from three individual experiments revealed a clear defect in activation of both signalling pathways, with a sharp initial induction of pERK/pAKT but a rapid decrease in these phosphoproteins compared to the parental line. Interestingly, the defect was identical in both *AFDN* and *SCRIB* KO cells. This implies that an AFDN-SCRIB complex or their proper localization at cell-cell contacts could be important for regulating the RAS-RAF or RAS-PI3K signalosomes. We used a luminescence-based cell viability assay to determine if altered signalling kinetics would impact proliferation, but EGF alone was not able to induce proliferation of the parental or KO lines. When we measured proliferation in complete media (10% serum) there was a small but significant reduction in proliferation of *SCRIB* KO cells, while *AFDN* KO cells proliferated at the same rate as the parental line (Figure 7F). As serum is not a specific RAS pathway activator, we can only conclude that loss of *AFDN/SCRIB* does not universally alter MCF7 proliferation.

Cancers driven by oncogenic RAS are characterized by predisposition to metastasis and malignancy. As AFDN and SCRIB are key regulators of adhesion and polarity we tested whether loss of *AFDN* or *SCRIB* would impact cell motility. In media containing serum we measured wound closure of MCF7, *AFDN* or *SCRIB* KO cells over a 72-hour time course (Figures S6C/D).

All cell lines could migrate into and close the wound, with no significant difference between their motility rates. We then attempted the same experiment but using only growth factor (EGF) as a more specific RAS-pathway activator (Figures 7G/H). Stimulation of MCF7 cells with EGF every 24 hours for a total of 96 hours resulted in near complete wound closure. Conversely, KO lines of *AFDN*, *SCRIB* or *AFDN/SCRIB* demonstrated a complete loss of motility in response to growth factor. We hypothesized that loss of adhesion and polarity may account for this defect, and immunostained cells at the leading edge with anti-GM130, a Golgi marker. Golgi will characteristically orient toward the wound in migrating cells, and Figure 7I shows this occurs in the MCF7 line. In contrast, both *SCRIB* and *AFDN* KO cells show randomly oriented Golgi that is not coordinated at all with wound direction. Thus, *AFDN-SCRIB* may be important mediators of adhesion and polarity that regulate the ability of RAS GTPases to promote motility.

Discussion

Binding of RAS GTPases to multiple effectors with diverse signalling properties allows these molecular switches to act as gatekeepers. Outside a subset of well-studied effectors (RAF, PI3K), however, the biological consequences of RAS GTPase interactions with most RA/RBD domain proteins remains unknown. It is particularly intriguing that proteomics approaches consistently identify AFDN as the most proximal RAS effector^{24,25}. Here, we have generated an AFDN interaction network and the combination of previously known and novel interactors provides a network library through which an AFDN-RAS complex might function. A top hit was the adhesion/polarity tumour suppressor protein SCRIB. Several lines of evidence suggest that loss of SCRIB function plays a role in metastatic conversion of RAS transformed cells. In *Drosophila*, where deletion of *scribbled* has become a leading model to study RAS oncogenesis^{47,59–63}, neoplastic tumours arise from cells with both *scrib* and *Ras* mutations⁴⁵. There are also substantial data linking SCRIB to regulation of the ERK/MAPK pathway in mammalian systems^{64–67}. A direct molecular path linking SCRIB to RAS, however, has never been previously elucidated. We show that RAS-G12V is co-localized with AFDN and SCRIB at sites of cell contact, while wild-type RAS is distributed more generally across the plasma membrane. Further, we characterized a complex comprising KRAS, its effector AFDN and SCRIB that could play an important role in the maintenance and stability of cell-cell contacts and polarity in cells harbouring activated RAS. Alternatively, in normal cells RAP GTPases may function to position AFDN-SCRIB at emerging sites of cell-cell adhesion to initiate and couple AJ formation to establishment of polarity.

In addition to SCRIB there are several noteworthy hits in the AFDN BioID dataset, many of which are tumour suppressors. The presence of the RA domain proteins ASPP2⁶⁸, RASSF8⁴² and ASPP1¹⁷ implies a dynamic network of RAS subfamily effectors at the cell membrane. The specificity of these proteins for GTPases has not been well defined, but their presence suggests that multiple RA domain effectors and multiple RAS GTPase 'switches' could read diverse inputs at subcellular domains like AJs, and transduce signals to the appropriate pathways dependent on GTPase-effector specificity and effector-associated protein domains. RASSF8 and the ASPP proteins are tumour suppressors proposed to function in the Hippo and p53 pathways, respectively^{69,70}. Other intriguing interactors include ROBO1, NOTCH2 and SLITRK4 that implicate AFDN in development and neurogenesis^{71–74}; PAK4 and ARHGAP21 which regulate

RAC1/CDC42 cytoskeletal remodelling⁷⁵⁻⁷⁷; DLG5, a membrane-associated guanylate kinase (MAGUK) protein involved in Hippo signalling^{78,79}; and numerous mitochondrial, Golgi and ER proteins which suggest a role for trafficking from the plasma membrane to these intracellular organelles.

The AFDN network is highly enriched in proteins with PDZ domains. These prevalent domains typically bind with moderate affinity to the C-termini of multiple targets, scaffolding a dynamic network of cell adhesion, polarity and cytoskeletal proteins. PDZ domains have evolved numerous mechanisms to perform these scaffolding functions, including binding to internal peptide motifs^{53,80}. Of the four PDZ domains in SCRIB, PDZ1 has considerably fewer binding partners than PDZ2-4 (β -PIX and *Drosophila* Gukh^{50,81}). The enrichment of PDZ domains in our proteomic dataset suggests one or more may bind AFDN in a similar manner to SCRIB. It is also noteworthy that the AFDN FHA domain has no previously recognized binding partners. The lack of positively charged ARG or LYS residues in this domain is strong evidence that it does not bind pTHR motifs and has instead evolved a unique binding mode. Related KIF family FHA domains also appear to lack phospho-dependency, and the kinesin KIF13 interacts with an adaptor CENPA1 independent of phosphorylation⁸². While we await elucidation of a structure for the AFDN-SCRIB complex, the non-canonical binding mechanisms highlight the complexity of studying protein-protein interactions based on predicted molecular functions.

Loss of function approaches are key to exploring the emerging connection between adhesion/polarity complexes and cellular transformation. To date, such experiments have provided a complex and inconsistent account of AFDN and SCRIB function. This includes numerous observations of both increased or decreased cellular proliferation, motility or invasion^{38,83-85}. This is likely due to both context and cell type dependence as well as the use of RNA-mediated knockdown approaches which typically retain a significant level of protein expression. Here, we have generated CRISPR knockout clones of MCF7 epithelial cells that completely lack AFDN or SCRIB expression. We observed little difference in the ability of these cells to proliferate or collectively migrate in the presence of serum, but significant defects in response to growth factor. EGF is a potent activator of RAS, and loss of AFDN or SCRIB disrupted MAPK and PI3K induction as well as migration. Unfortunately, we were unable to examine AFDN Δ FHA or SCRIB Δ PDZ1 rescue lines in these experiments (or wild-type) as expression of these proteins was rapidly inactivated within 24-48 hours in culture (Figure S6E).

This was observed independent of the tag or promoter used (CMV or EF-1 α). Further, re-expression of AFDN in *AFDN* KO cells was not able to rescue E-cadherin localization to AJs (Figure S6F-G), indicating there has been a re-programming of these cells that cannot be reverted by exogenous AFDN. This underscores the complex interplay of proteins at sites of cell-cell contact, whose positioning has a significant impact on gene expression and cell differentiation.

Future studies are required to elucidate comprehensive GTPase binding profiles for most proteins with RA or RBD domains, allowing systematic coupling of GTPases and effectors. Until then, it is essential to understand the biological processes impacted by specific effectors. Here, we've provided a network map for AFDN, a RAS GTPase effector that appeared early in the evolution of metazoans. The described interaction with the polarity protein SCRIB reveals a GTPase-AFDN-SCRIB axis that has implications for the formation and maintenance of cell adhesion during development, and the disruption of these processes during metastatic evolution in RAS-transformed cells.

Figure 1. BioID identification of proteins with *in vivo* proximity to AFDN.

(A) Activation of RAS subfamily small GTPases is governed by differential binding to guanine nucleotides. In the GTP-bound state RAS interacts with effector proteins including AFDN.

(B) Domain organization of the AFDN protein. RA=RAS association domain; FHA=forkhead-associated domain; DIL=dilute domain; PDZ= PSD95/DLG1/ZO-1 domain. These are followed by a proline rich region and a C-terminal helical domain involved in actin binding.

(C) High-confidence BioID hits (FDR ≤ 0.01) for both isoforms of AFDN were categorized by Gene Ontology (GO) Cellular Component (CC; top) or GO Biological Process (BP; bottom).

(D) The top 25 BioID preys with their associated domains. Available GO CC (square) or GO BP (circle) terms are colored as in (C). The relative abundance, FDR and spectral count are represented by shaded circles and denoted as per the legend (bottom).

See also Table S1 and Figure S1.

Figure 2. A SCRIB-AFDN complex is mediated by the FHA domain of AFDN and the PDZ1 domain of SCRIB.

(A) Localization of AFDN and SCRIB in HeLa cells. Venus-AFDN and immunostained FLAG-SCRIB were imaged by confocal microscopy. Scale bar represents 10 μ m.

(B) Endogenous SCRIB and AFDN co-localize at cell-cell contacts in MCF7 cells, as detected by immunostaining. Projections of z-stacks are at right and top of the merged images, with position shown by dashed yellow line. Scale bar represents 10 μ m.

(C) Full-length AFDN and SCRIB co-immunoprecipitate (IP). Expression vectors encoding GFP-tagged SCRIB and FLAG-tagged AFDN were co-transfected into HEK 293T cells. IP with anti-FLAG followed by Western blotting with anti-GFP revealed SCRIB complexed with AFDN. Vector alone was a control.

(D) Endogenous AFDN and SCRIB co-precipitate. MDCKII cells were grown to confluence and lysed. Following AFDN IP, an anti-SCRIB blot confirmed their complex. No AFDN or SCRIB were observed in an anti-Myc IP (control).

(E) Seven fragments encompassing whole AFDN were generated to map the interaction, comprised of the numerous AFDN modular domains.

(F) SCRIB is precipitated by the FHA domain of AFDN. GST-fusions of the 7 AFDN fragments were mixed with lysates expressing wild-type KRAS, KRAS-G12V, SCRIB, or the C and N-terminal regions of SCRIB. Full-length and endogenous SCRIB or the N-terminal half were bound by the AFDN FHA domain. Activated KRAS bound the RA domains.

(G) Fragments of SCRIB used for mapping its interaction with AFDN.

(H) AFDN was precipitated by the PDZ1 domain of SCRIB. GST-fusions of the SCRIB LRR or PDZ1 domains were mixed with lysates expressing either isoform of AFDN. Both isoforms and endogenous AFDN were bound by PDZ1.

(I) The AFDN-SCRIB complex is dependent on the FHA domain of AFDN. Full-length AFDN or an FHA deletion mutant tagged with GFP were co-transfected with FLAG-tagged SCRIB. Following anti-GFP IP, an anti-FLAG blot revealed SCRIB does not IP with AFDN Δ FHA.

(J) Complex between AFDN and SCRIB is dependent on the PDZ1 domain of SCRIB. Full-length or a PDZ1 deletion mutant of SCRIB tagged with GFP were co-transfected with FLAG-tagged AFDN. Following anti-GFP IP, anti-FLAG blot revealed SCRIB Δ PDZ1 does not precipitate with AFDN.

Figure 3. AFDN FHA and SCRIB PDZ1 domains interact directly in a phospho-independent manner.

(A) GST-FHA domains with differing C-termini can precipitate full-length SCRIB from cell lysate.

(B) Lack of positively charged residues on the surface of the AFDN FHA domain. The electrostatic surface of MDC1 FHA domain (*left*) shows the position of K73 and R58, necessary for binding pThr (PDBid 3UNN). Electrostatic surface of the AFDN FHA domain (PDBid 1WLN) reveals no positive charges at equivalent positions (*right*; dashed circle). Electrostatic potential was calculated using APBS and PyMol.

(C) AFDN and SCRIB co-IP following incubation with phosphatase. GFP-SCRIB and FLAG-AFDN were co-transfected and lysates precipitated with anti-FLAG. Western blotting with anti-GFP showed the interaction is not disrupted by phosphatase treatment.

(D) Purified SCRIB PDZ1 can interact directly with GST-tagged AFDN FHA domain. Fragments end at FHA domain residues 501-590 and could only bind SCRIB PDZ1 if extended past residue 580. Mixing with GST alone was a negative control.

(E) $^1\text{H}/^{15}\text{N}$ -HSQC spectra of the AFDN FHA domain. The extended FHA domain (371-580, black) overlays well with the core FHA domain (371-514, red) and exhibits several additional

peaks. The same peaks are broadened upon addition of unlabeled SCRIB PDZ1 (right, blue), verifying the importance of this extended C-terminal region to complex with SCRIB.

(F) The extended C-terminal FHA region alone does not bind SCRIB. Mixing the PDZ1 domain of SCRIB with GST-tagged FHA domain fragments revealed neither the core FHA domain (371-531) or C-terminal extension (531-580) in isolation can complex with PDZ1.

(G) ITC established the AFDN FHA domain binds the SCRIB PDZ1 domain with a K_d of 14.8 μ M, comparable to most PDZ domain interactions.

(H) Plot of peak intensity ratios from $^1\text{H}/^{15}\text{N}$ -HSQC spectra of PDZ1 alone vs PDZ1 in the presence of 2-fold molar excess AFDN FHA domain. Widespread broadening was observed, the most impacted are labelled by residue number (over 6X difference, dashed red line).

(I) Surface representation of the amino acids in SCRIB PDZ1 involved in binding the AFDN FHA domain (PDBid 5VWK). Residues demonstrating the most broadening are coloured red, moderate broadening in orange and no broadening in grey. Unassigned residues are white. Position of the β -PIX peptide is characteristic of PDZ interactions with C-terminal motifs.

See also Figures S2 and S3.

Figure 4. RAS GTPases complex with AFDN-SCRIB.

(A) Activated KRAS is co-precipitated with AFDN and SCRIB. HEK 293T cells were co-

transfected with vectors expressing FLAG-AFDN and GFP-KRAS-G12V (constitutively active). Both endogenous SCRIB and KRAS-G12V were detected following anti-FLAG IP. Transfection with FLAG vector alone served as a negative control.

(B) Activated KRAS and AFDN co-IP with SCRIB. HEK 293T cells were co-transfected with vectors expressing FLAG-SCRIB and GFP-KRAS-G12V. Endogenous AFDN and KRAS-G12V were detected following anti-FLAG IP.

(C) The RA domains of AFDN interact with multiple RAS small GTPases. GFP-tagged GTPases with activating mutations were expressed in HEK 293T cells, and GST-tagged RBD domains from BRAF or AFDN purified from bacteria. GST-RBDs bound to glutathione beads were used to pull down (PD) GTPases, a Western blot with anti-GFP revealed interacting proteins.

(D) Activated GTPases co-precipitate with AFDN and augment interaction with SCRIB. Full-length, GFP-tagged SCRIB and activated RAS subfamily GTPases were co-transfected with FLAG-tagged AFDN. Following immunoprecipitation with anti-FLAG, Western blot with anti-GFP revealed activated KRAS, RAP2C or RAP1B complex with AFDN and SCRIB.

(E) SCRIB lacking PDZ1 does not co-precipitate with AFDN and does not prevent the association between AFDN and KRAS. Full-length wild-type or Δ PDZ1 SCRIB were co-transfected with AFDN and KRAS. Association with SCRIB and KRAS was detected by Western blot following anti-FLAG immunoprecipitation of AFDN.

(F) Expression of wild-type KRAS does not alter AFDN or SCRIB localization. GFP-KRAS was expressed in MCF7 cells, and localization of endogenous AFDN and SCRIB was detected by immunostaining and imaging by confocal microscopy. Projections of *z*-stacks are at right and top. Position of associated *z* projections are shown with dashed yellow lines in the merged image. Scale bars represent 10 μ m.

(G) KRAS-G12V co-localizes with AFDN and SCRIB in MCF7 cells. Following expression of EGFP-KRAS-G12V, endogenous AFDN and SCRIB were detected by immunostaining. Cells expressing KRAS are found in the monolayer (1), detaching from the monolayer (2) or on top of the monolayer (3). Projections of *z*-stacks are at right and bottom of the merged images and their position is marked with a dashed yellow line. Scale bars represent 10 μ m.

See also Figure S4.

Figure 5. KO of *AFDN* or *SCRIB* disrupts cell-cell adhesion and complex formation.

(A) Individual clones of MCF7 cells were selected following CRISPR KO of *AFDN*, *SCRIB* or both *AFDN/SCRIB* with two different guide sequences. Western blot with antibodies recognizing endogenous AFDN or SCRIB confirmed the proteins are not expressed.

(B) Model of the SCRIB-AFDN-RAS GTPase complex based on our data.

(C) KRAS does not complex with SCRIB in the absence of AFDN. FLAG-SCRIB was expressed in *AFDN* KO MCF7 cells together with GFP-KRAS-G12V. Following IP with anti-FLAG, activated KRAS did not co-precipitate with SCRIB unless cells were also expressing GFP-AFDN.

(D) SCRIB is cytoplasmic in *AFDN* KO MCF7 cells, which do not form cell-cell contacts (top). Expression of GFP-KRAS-G12V in these cells does not alter SCRIB localization (bottom). Scale bars represent 10 μ m.

(E) AFDN remains predominantly at sites of cell contact in *SCRIB* KO MCF7 cells, which grow in multi-cell layers (top). GFP-KRAS-G12V expression results in AFDN internalization and breakdown of cell-cell contacts (bottom). Scale bars represent 10 μ m.

See also Figure S5.

Figure 6. Co-localization of SCRIB and AFDN is dependent on their PDZ1 and FHA domains, respectively.

(A) Expression of GFP-AFDN in *AFDN* KO MCF7 cells restores endogenous SCRIB localization at cell contacts. SCRIB is also recruited to cell contacts in *AFDN* KO cells expressing GFP-AFDNΔFHA but appears poorly co-localized with AFDN. Projections of z-stacks are at right and top and their position is marked with a dashed yellow line in the merged image. Scale bars represent 10 μm.

(B) GFP-SCRIB co-localizes with endogenous AFDN in *SCRIB* KO MCF7 cells. GFP-SCRIBΔPDZ1 is also enriched at cell contacts in *SCRIB* KO cells. Projections of z-stacks are at right and top and their position is marked with a dashed yellow line in the merged image. Scale bars represent 10 μm.

(C) z-stack projections along a 10 μm length of cell contacts show exogenous, wild-type AFDN

600 and endogenous SCRIB localization overlaps in *AFDN* KO cells (top, 3 independent cells).
601 Conversely, expressed *AFDN*ΔFHA remains apical to endogenous SCRIB (bottom).
602 **(D)** z-stack projections show exogenous, wild-type SCRIB and endogenous *AFDN* co-localize in
603 *SCRIB* KO cells (top) while *SCRIB*ΔPDZ1 remains basal to endogenous *AFDN* (bottom).
604 See also Figure S5 and S6.
605

Figure 7. Loss of *AFDN* or *SCRIB* disrupts ERK and AKT activation kinetics and cell motility in a growth factor-dependent manner.

Activation of the MAPK and PI3K pathways following EGF stimulation in MCF7 (A), *AFDN*

KO (**B**) or *SCRIB* KO (**C**) cells. pERK or pAKT Western blots measure pathway activity 2.5, 5, 15, 30 or 45 minutes after addition of EGF. Total ERK and AKT Western blots confirmed expression and serve as loading controls.

Quantification of MAPK (**D**) ERK activation) and PI3K (**E**) AKT activation) activity following addition of EGF over time. Error bars are derived from 3 independent replicates.

(**F**) Effect of *AFDN* or *SCRIB* KO on cell proliferation compared to parental MCF7 cells over a 72-hour time course. ** $P < 0.005$, ns = not significant, as measured by two-way ANOVA.

(**G**) KO of *SCRIB* or *AFDN* results in motility defects in response to EGF. Wound closure (%) was measured at 24, 48, 72 or 96 hours for MCF7 and *AFDN* KO, *SCRIB* KO, or *AFDN/SCRIB* KO cells. 100 ng/ml EGF was supplemented to the media at time 0 and every 24-hour interval thereafter. *** $P < 0.001$, * $P < 0.01$ as measured by RM one-way ANOVA (within group) or two-way ANOVA (between group).

(**H**) Phase contrast images of MCF7, *SCRIB* KO and *AFDN* KO cells during wound closure. 100 ng/ml EGF was supplemented to the media every 24 hours. Inset are enlarged images of cells at the wound edge.

(**I**) *AFDN* and *SCRIB* KO cells do not polarize towards the wound. Cells were stained for GM130 (Golgi), which typically orients towards the wound at the leading edge (see parental MCF7 cells; top). Cells were fixed 24 hours after wounding and EGF stimulation.

See also Figure S6.

Materials and Methods

Purification of Recombinant Proteins, Biochemical and Biophysical Protein Analysis, NMR Spectroscopy, Cell Culture and Immunoprecipitation, BioID-Mass Spectrometry, Phosphoproteomics, Proliferation Assay, MAPK and PI3K activity following EGF Stimulation, and In vitro wound healing assay are described in Supplemental Experimental Procedures.

Plasmid Constructs and Antibodies

For proteomic analysis we generated BirA*/FLAG-tagged mammalian expression constructs by Gateway cloning (Invitrogen) murine *Afadin* (GeneID 17356; residues 1-1820 for l-AFDN/iso1, or 1-1647 for s-AFDN/iso2) as entry vectors and then shuttling into BirA*/FLAG pcDNA5 FRT/TO. These were also placed in mammalian expression vectors with N-terminal FLAG or Venus tags. AFDN fragments were constructed in the same manner, as were full-length and fragments of human SCRIB (GeneID 23513; cDNA a kind gift from Dr. Senthil Muthuswamy (Beth Israel Deaconess Medical Center at Harvard)). Fragments of AFDN and SCRIB for interaction mapping or biophysical analyses were constructed by sub-cloning the indicated regions into pGEX-4T2 (Amersham Pharmacia Biotech) with N-terminal glutathione S-transferase (GST) tag for bacterial expression. Gateway entry vectors encoding mutationally activated human small GTPases KRAS (GeneID 3845), NRAS (GeneID 4893), HRAS (GeneID 3265), RAP1A (GeneID 5906), RAP1B (GeneID 5908), RAP2B (GeneID 5912), RAP2C (GeneID 57826), and DIRAS2 (GeneID 54769) were a kind gift from Dr. Jean-François Côté (IRCM, Montreal). These were shuttled into mammalian expression vectors with N-terminal Venus or FLAG tags. Point mutations and deletions were performed by PCR-directed mutagenesis or Gibson assembly. All constructs were verified by sequencing. Antibodies used were: anti-AFDN polyclonal (Abcam, ab203569), anti-AFDN monoclonal (Abcam, ab90809), anti-SCRIB (Cell Signalling, 4475S), anti- γ -Tubulin (Sigma, T6557), anti-Myc (Roche, 11667149001), anti-GFP (Abcam, ab290), anti-FLAG M2 (Sigma, F3165), anti-E-Cadherin (BD, 610181), anti-phospho-ERK1/2 (Cell Signalling, 9101), anti-phospho-AKT (Cell Signalling, 4060), anti-ERK1/2 (Cell Signalling, 4695) anti-AKT (Cell signalling, 4691), anti-mouse Tx-Red (Sigma, SAB3701076), anti-Rabbit Alexa Fluor 647 (Life Technologies, A-21244), anti-rabbit Cy5 (Abcam, ab6564), and anti-rabbit Alexa Fluor 488 (Life Technologies, A-27034).

Cell Imaging

For immunofluorescence, cells were split on ethanol sterilized coverslips in 6 well dishes and incubated at 37°C in 5% CO₂ for 24 hrs. To study co-localization of endogenous SCRIB and AFDN in the presence of KRAS, we transfected 1 µg of Venus, Venus-KRAS wild-type or Venus-KRAS-G12V into MCF7 cells using JetPrime (VWR). For co-localization of overexpressed SCRIB and AFDN in HeLa cells we co-transfected Flag-SCRIB and Venus-AFDN and immunostained to detect SCRIB. For immunostaining, cells were washed with PBS and fixed with 4% Paraformaldehyde (VWR) 48 hrs post-transfection. Permeabilization of cells was done in PBS containing 0.05% Tween-20 (PBS-T) and blocking with 4% FBS in PBS-T. Primary antibodies were diluted in blocking reagent and incubated with coverslips for 1 hr at 37°C in a humidified chamber (mouse anti-Flag (dil. 1/200), rabbit anti-SCRIB (Cell Signalling; dil.1/50) or mouse anti-l-AFDN (dil.1/100)). Coverslips were washed with PBS-T and incubated with secondary antibody for 1 hour at 37°C in a humidified chamber (goat anti-mouse-Tx-Red (dil.1/200), goat anti-rabbit-Cy5 (dil.1/300), goat anti-rabbit-Alexa Fluor 488 (dil.1/100), or Hoechst (dil.1/2000)). Following a final wash with PBS-T and ethanol, coverslips were mounted on slides with the Prolong Diamond antifade mountant (Life Technologies) and dried for 24 hr before acquisition. Imaging of the cells was performed using a laser scanning microscope LSM-880 (Zeiss). All images were taken with a 63x objective. 12 to 15 z-stacks were acquired (0.25 µm thickness) for each image and were merged by an XY orthogonal projection with the Zen lite 2.3 software (Zeiss). The following laser and detection wavelength were used: Hoechst (excitation 405 nm - detection 455/45 nm), Venus and Alexa Fluor 488 (excitation 488 nm - detection 525/25 nm), Tx-Red (excitation 561 nm - detection 602/28nm) and Cy5 (excitation 633 nm - detection 690/50 nm). To examine co-localization of SCRIB and AFDN we looked at z orthogonal projections in X or Y planes using Zen lite 2.3. Overlapping localization of SCRIB and AFDN in z orthogonal projections is interpreted as co-localization.

Generation of AFDN and SCRIB CRISPR KO cells and Rescues

AFDN and SCRIB MCF7 knockout cell lines were engineered using a CRISPR/Cas9 approach with the following guide RNAs (*SCRIB*: 5'-CACCGGAGAGCATCAAGTTCTGCA-3' and 5'-CACCGCTGGAGATCGCGGACTTCAG-3', both targeting exon 3 and *AFDN*: 5'-CACCGTTCCAGTGGTGGATGATGT-3', targeting exon 1 and 5'-

690 CACCGAGATGTAATCGAAACGCTCG-3', targeting exon 2). sgRNAs were designed using
691 the online tool at <https://zlab.bio/guidedesign-resources>. Each guide was cloned into the pSpCas9
692 (BB)-2A-Puro pX459 vector (Addgene #62988). Clonal cell lines were obtained by serial
693 dilution and isolation of colonies. Knockout was confirmed by immunoblotting. The precise
694 nature of the CRISPR/Cas9-mediated cleavage was assessed by Sanger sequencing. Four clones
695 were selected to make a reconstituted pooled population for *AFDN* KO and *SCRIB* KO. To re-
696 express *AFDN* and *SCRIB* in knockout cell lines, N-terminally GFP-tagged full-length wild-type
697 *AFDN*, *AFDN* Δ FHA, full-length wild-type *SCRIB* or *SCRIB* Δ PDZ1 were cloned into the
698 pLenti-CMV-GFP-Neo plasmid (Addgene #17447) using Gibson Assembly. Lentiviruses were
699 produced by co-transfection of vectors pMDLg/pRRE (Addgene #12251), pRSV-Rev (Addgene
700 #12253) and pMD2-VSVG (Addgene #12259) with GFP-*AFDN* or GFP-*SCRIB* pLenti plasmids
701 using calcium phosphate. HEK 293T cells were seeded in a 6-well plate 24 hrs prior to
702 transfection in complete DMEM medium (10% FBS), then transfected with the above-mentioned
703 transfection mixture. 24 hrs after transfection, medium was removed and replaced with fresh
704 medium (DMEM plus 5% FBS). 48 hrs post-transfection, virus containing medium was
705 harvested, filtered (0.45 μ m) and viral titer determined by infecting *AFDN* KO and *SCRIB* KO
706 pooled populations with a titration of *AFDN* and *SCRIB* lentivirus in the presence of polybrene
707 (8 μ g/ml). 72 hrs after infection, medium was replaced with fresh media containing 800 μ g/ml
708 G418 (Wisent, 400-130-IG) and cells were selected until 0% survival was observed in non-
709 infected controls. The multiplicity of infection (MOI) was determined 72 hrs post-infection by
710 comparing the percent of GFP fluorescence of infected cells to non-infected control cells. To
711 evaluate silencing the demethylating agent 5-Aza-2'-deoxycytidine (Sigma, A3656) was used at
712 20 μ M and the histone deacetylase (HDAC) inhibitor Trichostatin-A (AdooQ Bioscience,
713 A10947) at a concentration of 1 μ M or 0.2 μ M.

Acknowledgments

This work was supported by a grant from the Canadian Institutes for Health Research (CIHR, to M.J.S.). M.J.S. is also supported by the Cancer Research Society (CRS), the New Frontiers in Research Fund (NFRF) and the National Science and Engineering Council of Canada (NSERC). A.-C.G. is supported by a CIHR Foundation grant. M.J.S. holds a Canada Research Chair in Cancer Signalling and Structural Biology and A.-C.G. in Functional Proteomics. R.K. is supported by fellowships from the Cole Foundation and the Fonds de recherche du Québec-Santé (FRQS). S.S. is supported by scholarships from the Fonds de Recherche du Québec-Nature et technologies (FRQNT) and FRQS. Phosphoproteomics analyses were performed by the Center for Advanced Proteomics Analyses, a Node of the Canadian Genomic Innovation Network that is supported by the Canadian Government through Genome Canada

Author Contributions

This project was conceived and designed by M.J.S. M.G., M.J.S., V.G., S.S., R.K. and C.H.J. performed experiments and analyzed data. The manuscript was prepared by M.J.S. with input from V.G., R.K., S.S., M.G. and A.-C.G.

Competing interests: The authors declare no competing interests.

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