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Fundamental control of grade-specific colorectal cancer morphology by Src regulation of ezrin–centrosome engagement

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Abstract
The phenotypic spectrum of colorectal cancer (CRC) is remarkably diverse, with seemingly endless variations in cell shape, mitotic figures and multicellular configurations. Despite this morphological complexity, histological grading of collective phenotype patterns provides robust prognostic stratification in CRC. Although mechanistic understanding is incomplete, previous studies have shown that the cortical protein ezrin controls diversification of cell shape, mitotic figure geometry and multicellular architecture, in 3D organotypic CRC cultures. Because ezrin is a substrate of Src tyrosine kinase that is frequently overexpressed in CRC, we investigated Src regulation of ezrin and morphogenic growth in 3D CRC cultures. Here we show that Src perturbations disrupt CRC epithelial spatial organisation. Aberrant Src activity suppresses formation of the cortical ezrin cap that anchors interphase centrosomes. In CRC cells with a normal centrosome number, these events lead to mitotic spindle misorientation, perturbation of cell cleavage, abnormal epithelial stratification, apical membrane misalignment, multilumen formation and evolution of cribriform multicellular morphology, a feature of low-grade cancer. In isogenic CRC cells with centrosome amplification, aberrant Src signalling promotes multipolar mitotic spindle formation, pleomorphism and morphological features of high-grade cancer. Translational studies in archival human CRC revealed associations between Src intensity, multipolar mitotic spindle frequency and high-grade cancer morphology. Collectively, our study reveals Src regulation of CRC morphogenic growth via ezrin–centrosome engagement and uncovers combined perturbations underlying transition to high-grade CRC morphology.

Keywords: Src; ezrin; pole-like kinase; centrosome; mitotic spindle; cell division; phenotype; human; colorectal cancer; prognosis

Introduction
For over a century, histological grading of cellular and multicellular morphology has provided a robust readout of cancer aggressiveness [1]. Although causal molecular mechanisms are still unclear, fundamental and embryological studies provide important clues [2–4]. During development, evolution of tissue-specific architecture is coordinated by molecular interactions within the cell cortex, at the outer shell of the cell [2–4]. The cortex comprises the cell membrane linked to the underlying actin cytoskeleton meshwork by ezrin, radixin or moesin (ERM) proteins [5]. Ezrin recruitment from the cytosol to the cortex is aided by binding between the ezrin NH₂ terminal domain and phosphatidylinositol 4,5-bisphosphate (PIP₂) in the cell membrane [6]. Ezrin and other ERM molecules are then redistributed within the cortex to sustain mechanical stability [7,8] and to form a polarised accumulation called the ezrin cap, which provides a cue for centrosome anchoring [9]. These processes set bipolar symmetry for mitotic fidelity and control of emergent tissue properties [9,10].

Previous studies have uncovered spatiotemporal control of tissue assembly by crosstalk between the cortex and mitotic machinery [11–13]. Src family tyrosine kinases (SFKs) integrate cell cortex remodelling [11], alignment of the cell division axis [11], mitotic spindle dynamics [12] and 3D lumened multicellular assembly [13]. Src controls these important life processes by key signalling loops. A crucial event is Src phosphorylation
of ezrin at tyrosines (Y) 145 [14] and Y477 [15]. In particular, the Src interaction with ezrin Y145 enhances Src activity [14], which in turn, increases Akt signalling [16]. Akt phosphorylates ezrin at threonine (T) 567 [17] to promote wide ezrin conformational opening and binding to NHERF1 (also known as ezrin-binding protein 50; EBP50) [18], resulting in ezrin stabilisation at the cortex [19]. Subsequent cortical flow of ezrin during interphase leads to the formation of the polarised ezrin cap, which provides the cue for centrosome anchoring and establishment of mitotic symmetry [9,10]. Although this homeostatic machinery can be challenged by centrosome amplification induced by oncogenic polo-like kinase 4 (PLK4) [20], the ezrin cap clusters extra centrosomes [9,10], to conserve bipolar symmetry and evolution of regular 3D multicellular morphology [10].

To explore the molecular interplay underlying colorectal cancer (CRC) morphology, we dissected Src signalling crosstalk in CRC model systems. Because Src enhances Akt signalling by suppression of the tumour suppressor phosphatase and tensin homologue (PTEN) [16], we used stable isogenic PTEN-expressing and -deficient CRC cells to explore molecular interactions. We investigated the roles of ezrin phosphorytrosine domains at Y145 [14] and Y477 [15], as well as SFK specificity, in the control of ezrin cap formation. Because Src promotes aggressive cancer evolution from cells with extra centrosomes in a Drosophila model system [21], we investigated the interplay between Src, centrosome number and the evolution of multicellular morphology. We used isogenic Caco-2 CRC cells that predominantly have normal centrosome number, or a subclone where formation of extra centrosomes was induced by stable overexpression of PLK4. Using these models, we explored Src regulation of the morphogenic trajectory of a single CRC cell to lumenised multicellular architecture. We assessed sequential morphogenic processes, including ezrin cap integrity, centrosome positioning or clustering, mitotic spindle geometry and junctional protein dynamics. To explore clinical relevance, we conducted translational studies in archival human CRC.

This study addressed the provocative question of how subcellular processes guide evolution of grade-specific CRC morphology. We show that Src perturbation of ezrin cap formation in cells with a normal centrosome number leads to misorientation of the mitotic spindle. In turn, this process is causally implicated in the evolution of cribriform morphology [10,22], a feature of low-grade cancer [23]. Conversely, in cells with centrosome amplification, Src suppression of the ezrin cap drives asymmetric dispersal of extra centrosomes and the formation of multipolar mitotic spindles (MMS). These phenomena promote morphological features consistent with high-grade cancer [10]. Our translational studies support experimental findings and we found correlations between Src intensity, MMS frequency and high-grade morphology in human CRC. Collectively, the study provides a new molecular paradigm for the evolution of grade-specific CRC morphology by Src perturbation of ezrin–centrosome engagement.

Materials and methods

Reagents and antibodies
Most laboratory chemicals were purchased from Sigma-Aldrich (Poole, UK).

Cell lines
Stable PTEN-expressing Caco-2 and HCT116 PTEN+/− (PTEN+/+) and PTEN-deficient Caco-2 ShPTEN (ShPTEN) and HCT116 PTEN−/− (PTEN−/−) cells were raised or obtained and cultured as previously described [10]. Stable PLK4 overexpressing Caco-2 (Caco-2 PLK4OE) cells were generated as previously described [10].

Transfections
We carried out mammalian siRNA and plasmid DNA transfections using RNAiMAX and X-tremeGENE transfection reagents (Thermo Fisher, Dublin, Ireland), respectively, as previously described [10].

Confocal imaging
Assays of cell cortex dynamics, centrosome disposition, mitotic spindle orientation and geometry and multicellular patterns were conducted using a Leica SP5 confocal microscope (Leica Biosoftware, Milton Keynes, UK) with an HCX PL APO lambda blue 63× 1.40 oil immersion objective at 1x or 2x zoom, as previously described [10].

Human tumour samples
Anonymised formalin-fixed, paraffin-embedded (FFPE) colorectal primary tumours from previously described study cohorts [10] were released from the Northern Ireland Biobank, which has ethical approval to collect, store and distribute anonymised tissue samples to researchers by an approved protocol. Ezrin and Src immunohistochemistry (IHC) and quantification were conducted in the AstraZeneca Molecular Pathology Laboratory (Cambridge, UK). Full details of antibodies, siRNA oligonucleotides, plasmids, transfection reagents, cell culture methods, selection, confocal imaging, human tumour samples, IHC, immunofluorescence, ethical approval, reference numbers and data analysis are provided in supplementary material, Supplementary materials and methods.

Results

Src regulates ezrin via molecular crosstalk
To dissect molecular crosstalk underlying CRC morphology, we explored Src interactions with PTEN, using isogenic stable PTEN-expressing or -deficient Caco-2 and HCT116 CRC cells. We validated the Src kinase inhibitor AZD0530 [24] in dose/time course studies (see supplementary material, Figure S1A,B) and
selected the dose of 1 μM for all experiments. We conducted dominant-negative (DN) or constitutively active (CA) Src mutant transfections versus an empty vector only control (see supplementary material, Figure S1C). Here we found greater p-Src(Y416) expression in PTEN-deficient Caco-2 cells (Figure 1A,B). AZD0530 treatment suppressed p-Src(Y416) in Caco-2 and shPTEN cells and inhibited PTEN phosphorylation at S380/T382/T383 (p-PTEN(S380/T382/T383)) in Caco-2 cells (Figure 1A–C). EGF treatment activated Src [25] and increased p-ezrin(S380/T382/T383) in Caco-2 cells (Figure 1D–F). Results were similar in PTEN-expressing and -deficient HCT116 (PTENWT) and PTEN−/− in untreated conditions or after AZD0530 or EGF treatment, respectively (data not shown). Ezrin exists in multiple phosphorylation-dependent conformational states and p-ezrin (T567) is regarded as the active form [26]. Expression of p-ezrin (T567) was enhanced or suppressed by CA-Src and DN-Src transfection, respectively (Figure S1C,D). In 3D organotypic cultures of Caco-2 clones, we found maximal p-Src(Y416) subcellular localisation at plasma membranes and found higher intensity in shPTEN than in Caco-2 cultures (Figure 1G,H). AZD0530 treatment suppressed p-Src(Y416) intensity in 3D cultures (Figure 1G,H). Collectively, these studies uncovered functional PTEN/Src feedback signalling that controlled Src activity at cell membranes that in turn regulated ezrin phosphorylation.

Src regulates cell cortex dynamics

The cell cortex is an important regulator of cell fate and perturbations are fundamental to tumour growth and metastasis [27]. We and others have previously uncovered sequential processes of ezrin recruitment to the cell cortex from the cytosol at 3.5 h and subsequent ezrin cortical cap formation at 14 h, after synchronisation of cells in G0 [9,10]. To investigate Src regulation of these processes, we used phosphomimetic (E) and non-phosphorylatable (F) ezrin mutants at Src phosphorylation domains Y145 and Y377. Furthermore, we assessed the Src specificity of the effect by siRNA knockdown of the related SFKs, YES and FYN. We also modulated Src activity by transfection or treatment studies. We found accumulation of GFP-tagged wild type (WT) ezrin at the cortex at 3.5 h and formation of a polarised cap at 14 h after cell synchronisation. No cortical localisation or restriction of the GFP-tagged empty vector control was observed. The phosphomimetic GFP-tagged ezrin Y145E mutant also localised at the cortex and formed a cap more frequently than WT ezrin but the Y145 non-phosphorylatable mutant failed to localise or form a cap. However, neither phosphomimetic nor non-phosphorylatable mutations at ezrin 477 affected cortical recruitment or cap formation (Figure 2A,B). These results show that phosphorylation of ezrin at Y145 is crucial for ezrin redistribution from the cytosol to the cortex. Because ezrin is stabilised in the cortex by binding NHERF1 [19], we investigated the effects of AZD0530 treatment on cortical recruitment of each protein. AZD0530 treatment suppressed ezrin cortical recruitment at 3.5 h but did not affect cortical NHERF1 localisation (see supplementary material, Figure S2A, B; summary data for NHERF1 not shown). Although AZD0530 can suppress both Src family (e.g. c-Src, YES and FYN) and Abl tyrosine kinases [28,29], it has greater selectivity for the former, with more than 10-fold greater potency against Src than Abl [29]. To investigate SFK functional specificity, we conducted siRNA knockdown studies (see supplementary material, Figure S2,C,D). We found that siRNA knockdown of YES but not FYN suppressed ezrin cortical recruitment and cap formation (see supplementary material, Figure S2,E,F). These data show SFK functional overlap in relation to ezrin cortical dynamics. We then investigated the effects of Src overactivity by transfection of CA-Src versus DN-Src and empty vector control. In accordance with AZD0530 suppression of ezrin cortical recruitment (see supplementary material, Figure S2A, B), we found that DN-Src transfection suppressed ezrin cap formation (Figure 2C,D). Although CA-Src did not affect ezrin cap frequency (Figure 2C), it did affect ezrin cap phenotypes inducing multiple discrete ezrin accumulations within the cortex (Figure 2D) or increased intensity of p-ezrin(T567) localisation at the cap (see supplementary material, Figure S2,E,F) in approximately 30% and 32% of cells, respectively. Collectively, these studies implicate p-ezrin Y145 in cortical recruitment, show Src and YES functional overlap and uncover ezrin cap phenotypes resulting from Src overactivity.

Src regulates bipolar mitotic spindle orientation and multicellular assembly

Assembly of lumened colorectal glandular architecture is shaped by sequential processes of ezrin–centrosome engagement and bipolar mitotic spindle orientation [9,10,30]. To investigate Src regulation of spindle dynamics and multicellular assembly we conducted AZD0530 treatment studies in monolayer and 3D organotypic Caco-2 cultures. Here we found that AZD0530 treatment had a modest inhibitory effect on bipolar spindle formation (Figure 3A,B) but promoted spindle mis-orientation (Figure 3C,D) and aberrant multicellular assembly with the formation of multiple lumens (Figure 3E,F) in 4-day, 3D cultures. Although core processes of tissue assembly can be uncovered in short-term organotypic cultures, the evolution of cancer-relevant morphology often requires longer-term growth. At 12 days of culture, vehicle only treated control Caco-2 cultures formed regular glands comprising a uniform columnar epithelial monolayer arranged around a single central lumen. Conversely, AZD0530 treatment suppressed p-Src(Y416) signal intensity, induced epithelial stratification and multiple aberrant lumens consistent with cribriform multicellular morphology (see supplementary material, Figure S3A). In accordance with previous studies, we found that shPTEN cultures had
Figure 1. Src molecular interactions. (A) Effects of AZD0530 treatment on p-Src\(^{Y416}\) and phospho-PTEN [p-PTEN\(^{S380/T382/T383}\)] in Caco-2 and shPTEN cells. (B) p-Src\(^{Y416}\) densitometry values expressed as fold-change over vehicle only control [AZD0530 (−)] treatment in Caco-2; **\(p < 0.01\) and shPTEN cells *\(p < 0.01\). (C) p-PTEN\(^{S380/T382/T383}\) densitometry values expressed as fold-change over vehicle only control in Caco-2 cells; \(p < 0.05\); shPTEN; \(p = NS\). (D) EGF treatment effects on total Src and p-Src\(^{Y416}\) levels as well as total PTEN and p-PTEN\(^{S380/T382/T383}\) in Caco-2 and shPTEN cells. (E) Densitometry values for EGF effects on p-Src\(^{Y416}\), expressed as fold-change over vehicle only treated Caco-2 control; ***\(p < 0.001\); EGF shPTEN versus vehicle only shPTEN; *\(p < 0.05\). (F) Densitometry values for EGF treatment effects on p-PTEN\(^{S380/T382/T383}\) expressed as fold-change over vehicle only treated Caco-2 control; **\(p < 0.01\); EGF shPTEN versus vehicle only shPTEN; \(p = NS\). (G) Expression of p-Src\(^{Y416}\) in control or AZD0530 treated Caco-2 and shPTEN organotypic cultures at 4 days. At least 10 glandular structures were assayed in triplicate for each experimental condition. (H) Summary AZD0530 treatment effects on p-Src\(^{Y416}\) membrane intensity versus control in Caco-2; **\(p < 0.01\) and shPTEN; *\(p < 0.01\). GAPDH used as loading controls. Minor differences in magnification have been used to accommodate scale bars. Analysis by one-way ANOVA with Tukey’s post hoc test or paired Student’s t-test. Staining: DAPI for nuclear DNA (blue); anti-p-Src\(^{Y416}\) (red). Scale bar = 20 μm.

constitutively misoriented mitotic spindles relative to gland centres [10] (see supplementary material, Figure S3B,C). AZD0530 treatment did not significantly affect spindle orientation (see supplementary material, Figure S3B,C) or single central lumen formation in 3D shPTEN cultures (see supplementary material, Figure S3D). Taken together, these studies showed Src regulation of mitotic spindle orientation and...
multicellular assembly in PTEN-expressing cells. Conversely, spindle defects arising from PTEN deficiency were unaffected by Src inhibitory treatment.

**Src regulates bipolar spindle orientation via the focal adhesion complex**

During tissue assembly, bipolar spindle orientation is controlled by focal adhesion molecules focal adhesion kinase (FAK) and paxillin [31]. Although these proteins are important Src substrates, their role in Src regulation of bipolar spindle orientation and tissue morphogenesis remains unclear. FAK Y861 and paxillin Y118 are major Src phosphorylation sites [32,33], whereas FAK Y397 is an autophosphorylation site, activated by extracellular signalling [34]. Here we found that p-FAK(Y861) (Figure 4A,B) and p-paxillin(Y118) (Figure 4A) levels were suppressed by AZD0530 treatment. Conversely,
Figure 3. Src regulation of bipolar mitotic spindle dynamics and multicellular assembly. (A) Confocal images of spindle architecture in Caco-2 cells. (B) Summary treatment effects on bipolar spindle formation in Caco-2 and shPTEN cells (Caco-2 AZD0530 versus control; *p < 0.05; shPTEN AZD0530 versus control; p = NS). Values represent percentage of mitotic cells with bipolar spindles. At least 30 metaphase cells were assessed in triplicate for each experiment. (C) Treatment effects on mitotic spindle orientation and lumen formation in 4-day 3D Caco-2 glands. Insets (yellow box) show high-power views of spindle planes (solid white arrows) and their orientation relative to gland centres (GC, dashed white arrows). (D) Summary treatment effects on spindle orientation angles relative to gland centres in 3D Caco-2 cultures [AZD0530 (−) versus AZD0530 (+); ***p < 0.001]. (E) Summary treatment effects on single lumen formation in 4-day 3D Caco-2 cultures [AZD0530 (−) versus AZD0530 (+); **p < 0.01]. (F) Confocal images of treatment effects on lumen formation in 4-day 3D Caco-2 cultures. Minor differences in magnification accommodate marker of gland centres. At least 10 glandular structures were assayed in triplicate for each experimental condition. Staining: DAPI for nuclear DNA (blue); p-PKCζ for apical membrane marker (red); β-catenin for basolateral membrane. White arrows denote multiple lumens in AZD0530 treated cultures. Scale bar = 20 μm.
p-FAK Y397 levels were unaffected (see supplementary material, Figure S4A,B). Depletion of FAK by siRNA knockdown (see supplementary material, Figure S4C, D) led to mitotic spindle misorientation in 4-day Caco-2 organotypic cultures (Figure 4C,D). Taken together, these studies implicate focal adhesion complex molecules FAK and paxillin in Src control of spindle orientation and multicellular assembly.

Src regulation of supernumerary centrosome clustering and mitotic spindle architecture

Although bipolar spindle misorientation perturbs the plane of cell cleavage [35] and lumen formation [30], MMS formation devastates the spatial directives for multicellular assembly [10]. MMS frequently arises from centrosome amplification but clustering of extra centrosomes at the ezrin cap during interphase rescues bipolar spindle architecture [10]. To investigate relationships between Src signalling, centrosome amplification and spindle architecture, we used Caco-2 clones transfected with doxycycline-inducible PLK4 [10] and/or FLAG-tagged CA-Src (Figure 5A,B). Centrosome amplification was observed in 7.2 ± 1.18% (sem) parental Caco-2 cells and doxycycline induction of PLK4 in transfectants enhanced the frequency of extra centrosomes (Figure 5C). We observed clustering of extra centrosomes at spindle poles and rescue of bipolar spindle

![Figure 4](image-url)

**Figure 4.** Src regulation of bipolar spindle orientation via the focal adhesion complex. (A) Effects of AZD0530 treatment on FAK and paxillin phosphorylation in Caco-2 and shPTEN cells. (B) Summary treatment effects on p-FAK (Y861). Densitometry values AZD0530 (−) versus AZD0530 (+) in Caco-2; *p < 0.05; AZD0530 (−) versus AZD0530 (+) in shPTEN; **p < 0.01. (C) Effects of FAK siRNA knockdown (KD) on mitotic spindle orientation in 3D organotypic Caco-2 cultures at 4 days. Insets (yellow boxes) show high-power views of spindle planes (solid white arrows) and their orientation relative to gland centres (GC, dashed white arrows). (D) Summary effects of FAK siRNA transfection on spindle orientation angles in 4-day Caco-2 glands. Control versus FAK siRNA; ***p < 0.001. Student's t-test. At least 10 glandular structures were assayed in triplicate for each experimental condition. Staining: DAPI (blue), α-tubulin (green), pericentrin (red). Scale bar = 20 μm.
architecture both in WT Caco-2 cells and in PLK4 transfectants. CA-Src transfection suppressed centrosome clustering and enhanced MMS formation both in parental Caco-2 cells and in PLK4 transfectants (Figure 5C,D and see supplementary material, Figure S5A). In PLK4 transfectants, clustering of higher numbers of centrosomes was observed but dispersal induced by CA-Src led to more complex MMS formation, than in parental Caco-2 cells (see supplementary material, Figure S5A). To assess morphological consequences of MMS formation, we compared multicellular morphology of parental 3D Caco-2 cultures and in subclones with high MMS frequency induced by PLK4OE and suppression of ezrin cap formation. Minor differences in magnification accommodate the size differences between regular and irregular glandular structures. At least 10 glandular structures were assessed in triplicate for each experimental condition. Insets show bipolar and multipolar spindles. Staining: DAPI (blue), α-tubulin (green), p-PKCζ (red). Scale bar = 20 μm.
have bipolar spindles with a subclone with high MMS frequency. The MMS-high Caco-2 subclone was induced by stable PLK4 transfection and suppression of ezrin cap formation, in combination. We found that bipolar spindle architecture in parental 3D Caco-2 cultures associated with the formation of regular multicellular colorectal glandular structures with a single central lumen surrounded by a polarised columnar epithelial monolayer. Conversely, high MMS frequency associated with gross cytological and morphological disturbances including cellular and nuclear pleomorphism, misalignment of the apical membrane and loss of lumen formation (Figure 5E). Collectively, these results show that Src overactivity can suppress centrosome clustering and induce MMS formation that in turn promotes multicellular morphological perturbations, evocative of high-grade CRC.

Src association with spindle architecture in human CRC

To explore the clinical relevance of our in vitro studies, we investigated Src and ezrin expression using IHC on 35 human CRC and five normal colon tissue samples that had previously been characterised for apical NHERF1 expression, mitotic spindle architecture and histopathological prognostic features [10]. Src and ezrin IHC assays were optimised using normal human colon, then IHC intensities were assessed at three levels of intensity. Semiquantitative scoring of Src and ezrin expression was conducted using the H-score method (Figure 6A and see supplementary material, Supplementary materials and methods). Src intensity differed between distinct histological tumour grades (Figure 6B). Src and ezrin IHC intensities correlated (Figure 6C). Although Src intensity directly associated with the number of lymph node metastases in CRC (Figure 6D), ezrin intensity was unrelated to nodal metastases (see supplementary material, Figure S6A). Ezrin expression was greater in grade 3 versus grade 1 or 2 CRC but no difference was observed between grade 1 and 2 CRC (see supplementary material, Figure S6B). Furthermore, Src but not ezrin intensity directly associated with multipolar spindle formation (Figure 6E and see supplementary material, Figure S6C). Src and ezrin IHC intensities were unrelated to apical NHERF1 IHC intensity (data not shown). Collectively, these studies show the relationships between Src IHC intensity, MMS formation and high-grade multicellular morphology in human CRC.

Discussion

The elucidation of molecular circuits that control cancer cell growth to form distinct tumour type- or grade-specific morphological phenotypes has fundamental importance in pathology. Assembly of multicellular tissues is coordinated by the tumour suppressor PTEN and Src tyrosine kinase [13,36] through feedback signalling [16,37,38] and interactions with other key genes [14–17]. As the blueprint for lumened glandular architecture is set by ezrin cortical dynamics [9,10], a key question is how PTEN/Src crosstalk engages these processes. In the present study, we found inverse associations between PTEN and p-Src(Y416) expression in accordance with previous studies in breast cancer cell lines [38]. AZD0530 or EGF treatment or transfection by DN- or CA-Src, respectively, suppressed or enhanced Src kinase activity that in turn suppressed PTEN function [16]. Although not directly targeted by Src, we found that PTEN phosphorylation at S380/T382/T383 increased in proportion to Src activity. Phosphorylation at S380/T382/T383 promotes a more compact PTEN conformation that suppresses PTEN membrane binding and reduces its catalytic activity [39]. In 3D cultures, we found p-Src(Y416) localisation at cell membranes in accordance with previous cell monolayer studies [40] and found higher membrane p-Src(Y416) intensity in PTEN-deficient cells. Src is a member of a family of membrane-bound tyrosine kinases and membrane recruitment is enhanced by myristoylation [41]. AZD0530 treatment suppressed cell membrane p-Src(Y416) intensity in both PTEN-expressing and -deficient 3D cultures. These studies show functional PTEN/Src feedback and we investigated the role of this molecular crosstalk in ezrin dynamics.

Upon binding to cell membrane PIP2 [6], ezrin undergoes complex reconfigurations, including partial conformational opening [26]. Ezrin then becomes phosphorylated at specific tyrosines [14] and at T567 [42]. Akt [17] and other kinases [10,43–45] promote ezrin T567 phosphorylation that is required for binding NHERF1 [46], p-ezrin(T567) is widely used as a marker of the active ezrin state [26,42]. In this study, we have shown that p-ezrin(T567) is respectively enhanced or suppressed by CA-Src or DN-Src transfection. Although this effect could be linked to Src-enhanced Akt signalling [16] that promotes p-ezrin(T567) [17], Src is a promiscuous molecule and involvement of other kinases that phosphorylate ezrin at T567 [10,43,44] cannot be excluded.

To investigate the role of Src-driven ezrin phosphorylation in cortical dynamics, we transfected cells with phosphomimetic (E) and non-phosphorylatable (F) ezrin mutants at Src phosphorylation domains Y145 and Y477. We followed their intracellular distribution by confocal microscopy, focussing on ezrin cortical recruitment and cap formation at intervals of 3.5 h and 14 h after synchronisation in G0 [10]. We found that ezrin mutations at Y145 but not Y477 affected ezrin cap formation. Although the phosphomimetic ezrin Y145E enhanced cap formation, non-phosphorylatable ezrin Y145F abrogated ezrin cortical recruitment and generation of the cap. Here our results are in accordance with previous localisation studies of ezrin tyrosine mutants to the cell membrane [47]. How ezrin phosphorylation at Y145 regulates cellular localisation is not known. However, ezrin phosphorylation at this residue enhances binding to Src via SH2 domain interactions [14]. As Src is predominantly membrane-bound [41], this interaction could enrich ezrin at the cell membrane/actin.
Following ezrin’s cortical recruitment, it colocalises with and binds NHERF1, resulting in ezrin stabilisation [19]. Src affinity for ezrin [14] is important for ezrin subcellular distribution [47] but cortical localisation of NHERF1 was unaffected by Src activity. In siRNA knockdown and functional inhibition studies we found that Src and YES but not FYN regulated ezrin cortical recruitment and cap formation. The extensive sequence homology of these SFKs does not predict their peptide recognition preferences [48]. Src binds via its SH2 domain to a 13 residue (DNAMLEYLKAQD) peptide in the ezrin FERM domain [14,48] to phosphorylate ezrin at the critical Y145 residue implicated in growth control [14]. In large-scale screens of SH2 domain binding, Src was shown to share its peptide binding preference with YES but not with FYN [48] and may account for the SFK differences in ezrin regulation, shown in this study. To further investigate the role of Src in ezrin cap formation, we conducted transfection studies. In accordance with our findings of AZD0530 suppression of ezrin cortical recruitment, we found that DN-Src suppressed ezrin cap formation. Although transfection of CA-Src did not affect the frequency of ezrin cap formation, it had substantive effects on ezrin cap phenotypes, causing multiple ectopic ezrin accumulations (‘fragmented phenotype’) or formation of a ‘thickened’ cap with greater ezrin intensity, each in approximately 30% of cells. Although the functional significance of the thickened cap is unknown, each ezrin cortical accumulation within the fragmented phenotype can misposition centrosomes [9].

Figure 6. Src association with spindle architecture in human CRC. (A) Weak, moderate and strong immunostaining of Src and ezrin in archival CRC. (B) Src IHC intensity scores versus CRC grades 1–3 CRC; **p < 0.01 Wilcoxon signed rank test; grade 3 versus grade 1 or 2 **p < 0.01; grade 2 versus grade 1 or 3; *p < 0.01; ANOVA with Tukey’s post hoc test. (C) Src and ezrin IHC scores in archival CRC; Pearson’s r = 0.63; **p < 0.01. (D) Src IHC intensity scores versus number of lymph node metastasis. Pearson correlation = 0.379; p = 0.025. (E) Src IHC scores and MMS frequency in archival CRC; Pearson’s r = 0.442; **p < 0.01.
In physiological conditions, the interphase centrosome becomes anchored via astral microtubules to the cortical ezrin cap and then replicates [9]. Actin- and myosin II-mediated forces separate mother and daughter centrosomes to enable bipolar mitotic spindle formation [49]. Thus formed, the spindle is then orientated by mutually antagonistic apical and basolateral polarity forces [50]. Impaired cortical recruitment of ERM proteins [51] or defective cortical capture of astral microtubules [52] induce mitotic spindle misorientation [51,52] that has important consequences for cell cleavage [35], apical membrane alignment [30] and lumenised morphogenesis [30]. In this study, suppression of Src resulted in a modest reduction in bipolar spindle frequency but substantive bipolar spindle misorientation. Spindle misorientation associated with multilumen formation, epithelial stratification and evolution of a multicellular phenotype consistent with cribriform morphology, in 3D organotypic cultures. Cribriform morphology, characterised by multiple abnormal ‘back to back’ lumens surrounded by atypical stratified epithelium [53], is a feature of low-grade cancer and has a relatively favourable clinical outlook [23]. Bipolar spindle orientation is also regulated by PTEN [54] and in the present study PTEN-deficient cells showed frequent spindle misalignment. By phosphorylation of PTEN, Src drives a conformational change that impedes PTEN membrane binding but Src inhibitory treatment did not rescue morphogenesis of PTEN-deficient cells in this study. Collectively, our findings show that Src regulation of cortical dynamics in cells affects spindle assembly, bipolar spindle orientation and the formation of lumenised glandular architecture in 3D cultures of CRC cells with normal centrosome number.

Because mitotic spindle dynamics can be modulated by focal adhesion complexes [31], maintained by Src phosphorylation of FAK [32] and paxillin [33], we investigated Src regulation of those proteins in our model system. AZD0530 treatment suppressed phosphorylation of Src targets FAK Y861 and paxillin Y118 [32,33], but had no effects at FAK Y397, which is a FAK autophosphorylation domain [34]. Furthermore, siRNA knockdown of FAK promoted abnormal bipolar spindle orientation. Hence, our findings show that Src coordinates precise multimodal tissue assembly by regulation of bipolar spindle orientation, mediated in part by FAK phosphorylation.

Although Src has a central role in tissue homeostasis [11–13], stochastic increases in Src activity [55] or Src engagement of other oncogenes can perturb cell control mechanisms in cancerous tissues [56]. Highly conserved Src crosstalk with centrosomal kinases promote cancer hallmarks of invasion and metastasis [21]. To explore oncogenic co-dependency between Src and a centrosomal kinase, we conducted transfection of CA-Src and doxycycline-inducible PLK4 in Caco-2 cells. Most parental Caco-2 cells have a normal centrosome complement and as anticipated [20], PLK4OE induced substantial centrosome amplification. Parental Caco-2 cells with normal centrosome number formed bipolar spindles and the minority with extra centrosomes predominantly clustered them to rescue bipolar spindle formation. Transfection of Caco-2 cells with CA-Src did not affect centrosome number but suppressed centrosome clustering and enhanced MMS formation. Although PLK4OE induced substantive numbers of extra centrosomes, effective centrosome clustering and rescue of bipolar spindle formation was observed. However, the combination of PLK4OE and CA-Src induced dispersal of greater numbers of centrosomes and increased the frequency of MMS with complex configurations. Although bipolar spindle architecture directs the assembly of regular multicellular glandular structures, forced MMS formation promoted morphological features typical of high-grade cancer in organotypic cultures. Clinically, high MMS frequency in histological tumour sections associates with high grade in CRC [10] and poor outlook in CRC [10] and pancreatic cancer [57]. Although Src hyperactivity in combination with centrosome amplification promotes mitotic error, defective abscession and aberrant cytokinesis in 2D cultures [58], MMS-associated molecular signals that perturb 3D multicellular morphology and drive tumour progression have yet to be determined.

To investigate the clinical relevance of experimental findings, we investigated spindle architecture in archival human CRC. We found direct associations between high Src IHC intensity, multipolar spindle frequency and high-grade morphology. Furthermore, Src IHC intensity in CRC associates with regional lymph node metastasis. Hence, Src perturbation of basic processes of cortical remodelling and mitotic spindle assembly may have major prognostic significance in CRC. Collectively, the present study uncovers Src regulation of an interconnected repertoire of morphogenetic processes, including cell cortex rearrangements, mitotic spindle organisation, cell division and multicellular patterning. As well as acting as a transforming agent on its own, Src can also facilitate other oncogenic processes [59]. In this study, we have identified centrosome amplification as a cooperating partner of aberrant Src in the promotion of multipolar spindle formation and phenotypic attributes of high-grade CRC morphology.

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**Author contributions statement**

LR conducted signalling, transfection, treatment centrosome, spindle and multicellular morphogenesis assays, data analysis and figure preparation. RKD conducted signalling assays, some transfections, siRNA knockdowns, ezrin mutant transfections and supervised some morphogenesis assays. HK generated the ezrin 145 mutants and sequenced them and supervised some morphogenesis assays. SVS and CJE helped to design some experiments and critically reviewed the manuscript. FCC conceived the study, designed some experiments, analysed data and wrote the manuscript with additional input from all co-authors. All authors approved the final version of the manuscript.

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Supplementary materials and methods

Supplementary figure legends

Figure S1. Modulation of Src activity

Figure S2. Src regulation of cell cortex dynamics

Figure S3. Src regulation of bipolar mitotic spindle dynamics, multicellular assembly and gland lumen formation

Figure S4. Src regulation of focal adhesion proteins

Figure S5. Src/PLK4 co-dependent regulation of centrosome and mitotic spindle dynamics

Figure S6. Ezrin IHC scores and prognostic variables in human CRC