Tyrosine phosphorylation of LRP6 by Src and Fer inhibits Wnt/ β -catenin signalling

Qing Chen^{1,‡}, Yi Su^{1,‡}, Janine Wesslowski¹, Anja I Hagemann¹, Mirana Ramialison^{2,†}, Joachim Wittbrodt², Steffen Scholpp¹ & Gary Davidson^{1,*}

Abstract

Low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) function as transmembrane receptors to transduce Wnt signals. A key mechanism for signalling is Wnt-induced serine/threonine phosphorylation at conserved PPPSPxS motifs in the LRP6 cytoplasmic domain, which promotes pathway activation. Conserved tyrosine residues are positioned close to all PPPSPxS motifs, which suggests they have a functional significance. Using a cell culture-based cDNA expression screen, we identified the non-receptor tyrosine kinases Src and Fer as novel LRP6 modifiers. Both Src and Fer associate with LRP6 and phosphorylate LRP6 directly. In contrast to the known PPPSPxS Ser/Thr kinases, tyrosine phosphorylation by Src and Fer negatively regulates LRP6-Wnt signalling. Epistatically, they function upstream of β -catenin to inhibit signalling and in agreement with a negative role in regulating LRP6, MEF cells lacking these kinases show enhanced Wnt signalling. Wnt3a treatment of cells enhances tyrosine phosphorylation of endogenous LRP6 and, mechanistically, Src reduces cell surface LRP6 levels and disrupts LRP6 signalosome formation. Interestingly, CK1 γ inhibits Fer-induced LRP6 phosphorylation, suggesting a mechanism whereby CK1 γ acts to de-represses inhibitory LRP6 tyrosine phosphorylation. We propose that LRP6 tyrosine phosphorylation by Src and Fer serves a negative regulatory function to prevent over-activation of Wnt signalling at the level of the Wnt receptor, LRP6.

Keywords Fer; LRP6; Src; Wnt

Subject Categories Membrane & Intracellular Transport; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

Signalling by the Wnt family of secreted lipoproteins regulates cell proliferation and differentiation and has essential functions in

development and disease [1–3]. Wnts bind their principal transmembrane (TM) receptors Frizzled (Fz) with high affinity [4]. Depending on the presence of co-receptors, Wnt-Frizzled complexes activate β -catenin-dependent (canonical) as well as β -catenin-independent (non-canonical, such as planar cell polarity) pathways. Wnt/ β -catenin signalling requires low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6; *Drosophila* Arrow) as co-receptors [5–8].

LRP6 transduces Wnt/ β -catenin signalling through Ser/Thr phosphorylation of its five reiterated Pro-Pro-Pro-Ser-Pro (PPPSP) and adjacent casein kinase 1 (CK1) sites on its intracellular domain (ICD) (Fig 1A). These five dual phosphorylation sites are commonly referred to as PPPSPxS motifs. Phosphorylation of the PPPSP site involves multiple proline-directed kinases, including glycogen synthase kinase 3 (GSK3) and CyclinY/CDK14, in a cellular context-dependent manner [9,10]. Different members of the CK1 family phosphorylate the adjacent CK1 sites; however, the plasma membrane anchored CK1 γ is likely the key player [11]. Phosphorylation of CK1 sites lies downstream of, and requires, PPPSP site phosphorylation [9,11], although the reverse phosphorylation priming site model has also been proposed [12], reviewed in [13].

LRP6 PPPSPxS motif phosphorylation leads to inactivation of the β -catenin destruction complex via sequestration of Axin1 [9,11,14] as well as direct inhibition of GSK3 by the phosphorylated PPPSPxS motifs [15–17]. Exactly how the β -catenin destruction complex is inhibited remains controversial; however, suppression of β -catenin ubiquitination and the subsequent increase in free cytosolic β -catenin governs pathway activation [18]. Accumulation of β -catenin leads to its translocation to the nucleus and activation of Wnt target genes in combination with members of the Lef/Tcf family of transcription factors [19,20].

Although LRP6 has been reported to be tyrosine-phosphorylated [21], the relevance for LRP6 function and Wnt signalling remains unclear. Recent studies have implicated an LRP6 YxxYxxYxx Φ motif with AP2 interaction [22] as well as with a negative regulatory role for LRP6/Wnt signalling [23]. This motif is claimed to regulate LRP6 endocytic trafficking [23]. In spite of these indications that tyrosine phosphorylation and tyrosine-based motifs play an important role in

¹ Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

² COS Heidelberg, Heidelberg University, Heidelberg, Germany

*Corresponding author. Tel: +49 721 608 26103; E-mail: gary.davidson@kit.edu

[†]Present address: Australian Regenerative Medicine Institute, Monash University, Clayton Campus, Clayton, Vic., Australia

[‡]Equal contribution

A LRP6 intracellular domain

Human	GSLPGMSRGKSMISL---SIMGSSGPPYDRAHVTGASSSSSSTKGYFPAILNPPPSPATERSHYTMEFGYS
Mouse	GSLPGMSRGKSMISL---SIMGSSGPPYDRAHVTGASSSSSSTKGYFPAILNPPPSPATERSHYTMEFGYS
Xenopus	-----GSL---SIMGSSGPPYDRAHVTGASSSSSSTKGYFPAILNPPPSPATERSHYTMEFGYS
Zebrafish	GSLPGMSRGKSMISL---SIMGSSGPPYDRAHVTGASSSSSSTKGYFPAILNPPPSPATERSHYTMEFGYS
Drosophila	-----RVSKIASVADAVRMSTLNSRNSMNSYDRNHITGASSTNGSSMVAIP--INPPSPATRSRR-----
	1460 1480
Human	SNSPSTHRSYSYRPSYRHFAPPTTPCSTDVC---DSDYAP-----SRR---MTSVATAKGYISDLNYDSEP
Mouse	SNSPSTHRSYSYRPSYRHFAPPTTPCSTDVC---DSDYAP-----SRR---MTSVATAKGYISDLNYDSEP
Xenopus	SNSPSTHRSYSYRPSYRHFAPPTTPCSTDVC---DSDYTP-----SHR---LTSAAAAGKGYISDLNYDSEP
Zebrafish	SNSPSTHRSYSYRPSYRHFAPPTTPCSTDVC---DSDYTP-----GRRAPHASSATAAKGYASDLNYDSEP
Drosophila	-----PYRHXKIINQPPPTPCSTDICDESNSYTSKNSNNSNGGATKHSSSSAAA---CLQYGYDSEP
	1517 1520 1541 1562
Human	VPPPTTPRSQYLSAEE--NYESCPSFYTERSTSHHLYPPPPSPCTDSS---
Mouse	VPPPTTPRSQYLSAEE--NYESCPSFYTERSTSHHLYPPPPSPCTDSS---
Xenopus	VPPPTTPRSQYLSAEE--NYESCPSFYTERSTSHHLYPPPPSPCTDSS---
Zebrafish	LPPPTTPRSQYLSAEE--NCESCPSFYTERSTSHQLYPPPPSPCTDSS---
Drosophila	YPPPTTPRSQYLSAEE--SDVRIVPESSCPSFSRSTTFSPPLPPPPSPVQSPSRGFT
	1577 1597

B **C** **D** **Figure 1. Identification of Src and Fer as LRP6 tyrosine kinases.**

A Multiple sequence alignment (Clustal W) showing evolutionarily conserved tyrosines within the ICD of LRP6 (red). The CK1 cluster (dark gray) and PPPSP sites (light gray) are highlighted for reference.

B Overview of Medaka kinase cDNA library expression screen that identified Src and Fer. See Materials and Methods for details.

C Western blots of immuno-purified proteins after an *in vitro* kinase assay, showing direct tyrosine phosphorylation of LRP6 by Src. A pan-phospho-tyrosine antibody (panYp) was used to specifically detect tyrosine-phosphorylated LRP6.

D TOPFLASH reporter assay (upper graph) and Western blots (lower graph) of lysates from HEK293T cells transfected with the indicated genes in 96-well format. Wild-type (wt) Src, but not a kinase dead (kd) form of Src (K298M), promotes LRP6 tyrosine phosphorylation and inhibits LRP6-mediated Wnt signalling. Amounts transfected: *hLRP6*, 20 ng; *hSrc*, 10 ng. Data represent mean $\pm$ SD. ***P* < 0.01.

the regulation of LRP6 function, no LRP6 tyrosine kinases have yet been identified.

Src and Fer are non-receptor tyrosine kinases that regulate cell proliferation, differentiation and cell–cell adhesion. Fer knockout mice display minor defects in cortactin phosphorylation and immune cell development [24,25]. Src knockout mice develop osteopetrosis, have defects in T-cell development and have impaired memory [26]. Src and Fer have been reported to positively regulate Wnt/ β -catenin signalling via direct phosphorylation of β -catenin, which releases it from cadherin complexes at the membrane (reviewed in [27]). In addition, Src has been reported to associate with Dvl upon Wnt stimulation and positively regulate Wnt/ β -catenin signalling [28]. In *C. elegans*, Src has also been shown to promote Wnt signalling through phosphorylation of the β -catenin homolog, HMP-2 [29]. Conversely, Fer has been demonstrated to function as a negative regulator of this pathway in *C. elegans* by maintaining membrane localization of HMP-2 [30]. No LRP6 homolog has yet been identified for *C. elegans*.

Here, we demonstrate that both Src and Fer phosphorylate LRP6 and that this negatively regulates Wnt/ β -catenin signalling. Distinct mechanisms account for this inhibition, which suggests LRP6 activity is attenuated by a dual tyrosine phosphorylation process. We show that Wnt stimulates tyrosine phosphorylation of LRP6 and our data suggest a new feedback model in Wnt receptor activation and may help explain the known crosstalk between non-receptor tyrosine kinases and Wnt signalling.

Results

Identification of Src and Fer as regulatory LRP6 kinases

Multiple sequences alignment of LRP6 from five different species reveals 8 evolutionary conserved tyrosine residues in the intracellular domain (ICD) (Fig 1A). This suggests that tyrosine phosphorylation of LRP6 plays a functional role in Wnt signalling. To identify potential LRP6 tyrosine kinases, we performed a cell culture-based expression screen where we co-expressed LRP6 with cDNA clones from a kinase cDNA library (Fig 1B, see Materials and Methods for details). LRP6 Western blot screening of cellular lysates using a pan-phospho-tyrosine antibody identified two potential LRP6 tyrosine kinases, Src and Fer (Fig 1B). We performed *in vitro* kinase assays using immuno-purified proteins to confirm the directness of these phosphorylation events on LRP6 for both Src (Fig 1C) and Fer (Supplementary Fig S1A). We next analyzed the functional consequence of LRP6 tyrosine phosphorylation on Wnt/ β -catenin signalling using the established TOPFLASH reporter. In contrast to the well-documented Ser/Thr phosphorylation events at PPPSPxS motifs that activate signalling [9,11], both Src- and Fer-mediated LRP6 phosphorylation inhibit Wnt-LRP6 signalling (Fig 1D; Supplementary Fig S1B). These effects are dependent on the kinase activity of Src and Fer since kinase dead mutants neither phosphorylate LRP6 nor inhibit the TOPFLASH reporter (Fig 1D; Supplementary Fig S1B). A dose-dependent inhibition of Wnt signalling coincides with increased LRP6 tyrosine phosphorylation for both Src and Fer (Fig 2A; Supplementary Fig S1B). This inhibition of Wnt signalling activity occurs despite a parallel dose-dependent increase in total LRP6 protein levels (Fig 2A; Supplementary Fig S1B).

For Src and Fer to phosphorylate LRP6, they need to interact with LRP6 and this interaction was confirmed by co-immunoprecipitation experiments for both Src (Fig 2B) and Fer (Supplementary Fig S2A). The non-related transmembrane receptor FLRT shows no interaction with either Src or Fer, thus confirming specificity. Epistasis experiments are in agreement with Src and Fer functioning at the level of LRP6 because inhibition could be observed when the pathway was activated with Wnt, LRP6 and dishevelled (Dvl) but not β -catenin (Fig 2C; Supplementary Fig S2B). This indicates that Src and Fer function upstream of β -catenin in the Wnt pathway to inhibit signalling. We conclude that Src and Fer directly phosphorylate LRP6 on tyrosine residues and that this results in the inhibition of LRP6 signalling activity.

Src inhibits LRP6 signalling in developing zebrafish embryos

In order to investigate the role of Src in a developmental context, we performed experiments in zebrafish embryos, where Wnt/ β -catenin signalling regulates anteroposterior (a-p) patterning [31] (Fig 2D). While injection of *Src* mRNA has no overt phenotypic effect on the development of zebrafish embryos (Fig 2D, *Src*), *LRP6* mRNA injection resulted in a reduction in the size of the eyes (Fig 2D, *LRP6*), which is characteristic of the posteriorization phenotype expected upon activation of Wnt/ β -catenin signalling. Importantly, co-injection of *Src* mRNA rescued this *LRP6* gain-of-function phenotype (Fig 2D, *LRP6* + *Src*), demonstrating that Src can also inhibit LRP6 in the context of a developing embryo. Supporting this, *in situ* hybridization of zebrafish embryos for the direct Wnt/ β -catenin target gene, *Axin2*, showed significant downregulation upon injection of *Src* mRNA (Supplementary Fig S2C).

Multiple tyrosine residues are relevant for Src-mediated LRP6 inhibition

Considering that there are a total of eight evolutionarily conserved tyrosine residues spread throughout the ICD of LRP6 (Figs 1A and 3A), we first attempted to narrow the search for relevant sites by using LRP6- Δ E1–4- Δ 87, which lacks most of the ECD [32] as well as the last 87 residues of the ICD [11]. The Δ 87 ICD deletion removes four of the five PPPSPxS motifs as well as four of the eight conserved tyrosine sites (Fig 3A, Δ 87). We compared Src-induced LRP6 tyrosine phosphorylation levels for this Δ 87 deletion construct with wild-type (wt) LRP6 Δ E1–4, which contains a complete ICD and which generates a robust Src-induced phospho-tyrosine (panYp) signal (Fig 3A, panel Δ E1–4, wt). Src induces a clear phospho-tyrosine signal using the Δ 87 mutant, indicating that the more N-terminally located ICD tyrosine residues in this construct are phosphorylated by Src, although the reduction in signal suggests that Src likely targets tyrosine residues located more C-terminally, missing in the Δ 87 construct.

In order to further help pinpoint relevant tyrosine residues, we performed mass spectrometry (MS) analysis on Flag-LRP6 immunopurified from lysates of cells expressing either LRP6 alone or LRP6 co-expressed with Src (Supplementary Fig S3A). In the absence of overexpressed Src, a significant level of phosphorylation is detected at several tyrosine residues, including the conserved position Y1520

Figure 2. Src interacts with LRP6 and inhibits Wnt/β-catenin signalling.

- A** TOPFLASH reporter assay (upper graph) and Western blots (lower graph) of lysates from the same HEK293T cells transfected as indicated in 96-well format. Note that Src phosphorylates LRP6 and inhibits Wnt/β-catenin signalling in a dose-dependent manner. Amounts transfected: *hLRP6*, 20 ng; *mdSrc*, as indicated. Data represent mean $\pm$ SD.
- B** Western blots of immunoprecipitates (IP) or initial lysates (lysate) from HEK293T cells transfected as indicated in 6-well format. The transmembrane receptor FLRT serves as a negative control. Amounts transfected: *hF-LRP6*, 600 ng; *hF-FLRT*, 600 ng; *hSrc*, 300 ng.
- C** TOPFLASH reporter assays of lysates from HEK293T cells transfected as indicated or treated with Wnt3a-conditioned medium (Wnt3a CM) in 96-well format. Amounts transfected: *hSrc*, 5 ng; *hLRP6*, 20 ng; *hDVL1*, 20 ng; *Xtβ-catenin*, 2.5 ng. Data represent mean $\pm$ SD. n.s., not significant; ** P < 0.01.
- D** Head region of 24 h post-fertilization zebrafish embryos microinjected with *src* mRNA or *lrp6* mRNA as indicated. A red dashed line delineates the area of the eye on one side of each embryo. Amounts injected: *hLRP6-EGFP*, 2 ng; *mdSrc*, 0.2 ng per embryo. The penetrance of the phenotype shown (in %) and number (*n*) of embryos analyzed are indicated.

(Fig 3B; Supplementary Fig S3). This is in agreement with the low level of LRP6 tyrosine phosphorylation detected on the purified Flag-LRP6 protein by the pan-phospho-Yp antibody, in the absence of co-expressed Src (Supplementary Fig S3A, panYp blot) and demonstrates that LRP6 is indeed subject to tyrosine phosphorylation by endogenous cellular tyrosine kinases. Src co-expression generally increases the phosphorylation of these already phosphorylated sites; however, it also induces robust phosphorylation at the otherwise non-phosphorylated conserved sites Y1460 and Y1480 (Fig 3B; Supplementary Fig S3, see Materials and Methods for details). No phosphorylation was detected at the conserved site

Y1517 or site 1522 ($^{1522}\text{Yxx}\Phi$ motif of vertebrate LRP6). These results confirm that Src can phosphorylate multiple tyrosine sites within the ICD of LRP6.

Next, to identify which sites play a greater functional significance with respect to signalling inhibition, we generated tyrosine to phenylalanine (Y to F) point mutants at 6 sites within full-length LRP6 (Fig 3B, sequence below graph). Four of these correspond to evolutionarily conserved sites and are highlighted in red in Fig 3B and C. Using these point mutants, we tested the ability of Src to: (1) induce a tyrosine phosphorylation signal (Fig 3C, panYp) and (2) inhibit their TOPFLASH signalling activity

Figure 3. Src phosphorylates multiple tyrosine residues in LRP6.

A Left panel: Schematic representation of full-length (FL) or ΔE1-4 FLAG LRP6 proteins with intact and C-terminally deleted ICDs. TM, transmembrane domain; WT, wild-type. Right panel: WB of lysates from HEK293T cells transfected with the indicated LRP6 constructs, with or without co-transfection of Src, in 96-well format. Amounts transfected: *hwt*-LRP6 and *hΔE1-4*-LRP6 constructs, 20 ng; *mdSrc*, 2.5 ng.

B Quantification of phosphorylated LRP6 peptides at the indicated tyrosine (Y) residue within the ICD of LRP6, after LC-MS-MS analysis of h-Flag-LRP6 from HEK293T cells transfected with F-LRP6 or F-LRP6 + Src (and see Materials and Methods for details). A Schematic representation of the position of the conserved tyrosines is shown beneath. Data represent mean ± SD.

C Western blots of lysates from HEK293T cells co-transfected with hSrc (10 ng) and the indicated LRP6 constructs (20 ng) in 96-well format. The LRP6 mutant constructs bear single or combined tyrosine (Y) to phenylalanine (F) mutations at the indicated residue(s).

D TOPFLASH reporter assay using lysates of HEK293T cells transfected with the indicated LRP6 mutants shown in (C), in 96-well format, with (black bars) or without (white bars) co-transfected Src. Amounts transfected: as in (C). Data represent mean ± SD. Note that only the combinatory Y4F mutant form of LRP6 is statistically different from wt LRP6 in terms of Src-mediated inhibition. **P* < 0.05.

Figure 4. Wnt3a induces LRP6 tyrosine phosphorylation.

A WB of lysates from MEF cells transfected in 6-well format with 25 pmol of control (non-targeting) siRNA or siRNA targeting human LRP6 and treated with or without Wnt3a-conditioned medium for the indicated times immediately prior to harvest. Endogenous membrane LRP6 was then enriched by membrane protein fractionation. Wnt3a-induced phosphorylation of endogenous LRP6 was detected using a phospho-tyrosine-specific LRP6 antibody (Yp1460, see Materials and Methods for details) or a PPPSP phospho-specific LRP6 antibody (Sp1490). Total LRP6 protein levels were detected using the total LRP6 specific T1479 antibody.

B TOPFLASH assay (upper graph) and Western blots (lower panels) of lysates from wild-type (wt) or homozygous mutant (–/–) SYF MEF cells transfected as indicated in 24-well format and treated overnight with Wnt3a-conditioned medium as indicated. 100 ng of mSrc was transfected for lanes 5 and 6. β-actin was used to normalize for protein loading.

(Fig 3D). Mutation of the 4 more N-terminally located sites Y1460, Y1480, Y1517 and Y1520 results in significant reduction of Src-induced tyrosine phosphorylation (Fig 3C). Indeed, combining these mutations (1460 + 1480 + 1517 + 1520 - "Y4F") eliminates the pY signal to almost undetectable levels (Fig 3C, YF4). With

respect to their relevance for Wnt signalling, the individual Y1480 and Y1520 point mutants reduce the inhibitory effect of Src; however, the YF4 mutant is not significantly inhibited by Src (Fig 3D). These results confirm that Src-mediated LRP6 inhibition is a result of LRP6 tyrosine phosphorylation and we conclude that multiple tyrosine residues are functionally relevant Src phosphorylation sites that inhibit LRP6 activity.

Wnt3a stimulates LRP6 tyrosine phosphorylation

Previous studies have demonstrated that the PPPSPxS motifs of LRP6 are phosphorylated upon Wnt stimulation [9–11,14]. We therefore investigated whether LRP6 tyrosine phosphorylation is also regulated in a Wnt-dependent manner. As expected, Wnt3a, when supplied to untransfected cells as conditioned medium, stimulates endogenous LRP6 PPPSP phosphorylation as detected by a phospho-specific antibody that recognizes the first PPPSP motif (Fig 4A, Sp1490 panel), [14]. Strikingly, a time-dependent increase of endogenous LRP6 tyrosine phosphorylation is also seen, as detected by a specific antibody we raised that recognizes LRP6 phosphorylated at tyrosine residue Y1460 (Fig 5A, Yp1460 panel). The lack of an Yp1460 Antibody signal in lysates from cells treated with LRP6 siRNA confirms specificity (Fig 5A).

To address the effect of endogenous Src family kinases on Wnt/β-catenin signalling and LRP6 tyrosine phosphorylation, we performed experiments using mouse embryonic fibroblasts lacking the Src family kinases Src, Yes and Fyn (SYF^{-/-} MEF cells) [24]. Wnt-induced LRP6 tyrosine phosphorylation is also seen in SYF wt cells (Fig 4B, lanes 1–2, Y1460 panel) and, demonstrating that Src is required for this, the phosphorylation signal is absent in the corresponding SYF mutant cells (Fig 4B, lanes 3–4, Y1460 panel). Reintroduction of Src rescues the phosphorylation event, confirming specificity (Fig 4B, lanes 5–6, Y1460 panel). Lysates from the same cells were also subjected to Wnt reporter assays. Compared to wild-type cells, a striking enhancement in Wnt reporter activity is seen in mutant cells in response to Wnt3a stimulation (Fig 4B, upper graph, compare lanes 2 and 4). This enhanced sensitivity to Wnt3a stimulation is specifically due to loss of Src because the effect is rescued by reintroduction of Src (Fig 4B, upper graph, compare lanes 4 and 6). For loss of function in MEF cells also results in Wnt/β-catenin signalling sensitization, although to a lesser degree than SYF cells (Supplementary Fig S4A). We also tested the effect of a more acute loss of Src in HEK293 cells using the chemical inhibitor PP2. Treatment of cells for 1 h with PP2 results in slight inhibition of basal Wnt signalling, in agreement with our finding that Src overexpression usually promotes basal signalling (e.g. Fig 2A). Indeed, SYF^{-/-} cells also have reduced basal Wnt signalling (Fig 4B). In contrast, a slight enhancement of signalling is seen if cells are treated with Wnt3a-conditioned medium (Supplementary Fig S4B). Although this sensitization of HEK293 cells to Wnt stimulation upon PP2 treatment is not as strong as for SYF^{-/-} MEF cells, the results are in agreement. Similar to the situation for Wnt3a treatment, we also see an enhanced PP2-mediated induction of TOPFLASH signal if cells are co-transfected with LRP6 (Supplementary Fig S4C). Taken together, these results confirm that Src and Fer are novel LRP6 tyrosine kinases that function to negatively regulate Wnt/β-catenin signalling.

Figure 5. Src inhibits LRP6 signalosome formation.

A Biotinylation and Western blot analysis of endogenous LRP6. HEK293T cells were transfected in 6-well format with 1 μg mdSrc or LacZ (Con.) as indicated. Cell surface proteins were biotinylated and precipitated with avidin-agarose beads before immunoblotting (see Materials and Methods for details).

B LRP6 aggregation analysis. Western blot of lysates from HEK293T cells transfected as indicated in 10-cm plates with 600 ng LacZ (con) or hSrc (+Src) and treated with control (–) or Wnt3a-conditioned medium (+) for 3 h prior to harvest. Harvested lysates were carefully subjected to Opti-Prep density gradient centrifugation to separate the different molecular weight species of LRP6 into different fractions, with fraction 1 harboring the highest-molecular-weight protein aggregates and fraction 14 the lowest-molecular-weight forms. Aliquots of each fraction were analyzed by SDS-PAGE/WB for the abundance of total (T1479) or PPPSP site phosphorylated (Sp1490) LRP6.

C Confocal microscopy analysis of live zebrafish embryos injected with 1 ng of *Wnt8-GFP*, 2 ng of *LRP6-mCherry* and, where indicated, with 2 ng of medaka *Src* mRNA at the one-cell stage and imaged at 30–50% epiboly. Arrowheads indicate Wnt-LRP6 clusters on the plasma membrane (LRP6 signalosomes). Note that *Src* co-injection results in disruption of LRP6 aggregates, although Wnt remains localized at discrete puncta on the membrane. Scale bar is 15 μm.

D Quantification of the colocalization between Lrp6 and Wnt8 in confocal microscopy analysis shown in (C). *Src* co-expression leads to a lower quantity of Lrp6 colocalizing with Wnt8, represented by the Mander's colocalization coefficient. Data represent mean ± SD.

Figure 6. CK1 γ inhibits Fer-mediated LRP6 phosphorylation.

A TOPFLASH reporter assay from lysates of HEK293T cell transfected as indicated in 96-well format. Amounts transfected: *hLRP6*, 20 ng; *mdFer*, 4 ng; *XtCK1γ*, 2 ng.

B Western blots of lysates from HEK293T cells transfected with indicated genes in 96-well format. Amounts transfected: *hLRP6*, 20 ng; *mdFer*, 4 ng; wt *XtCK1γ*, 2 ng; kd (*K73R*) *XtCK1γ*, 15 ng. Fer and CK1γ-induced LRP6 phosphorylation were detected by pan-phospho-tyrosine or CK1 sites phospho-specific Tpl479 antibodies, respectively. Antibody T1479 recognizing total LRP6 was used as an input control for LRP6.

Src inhibits LRP6 by reducing its cell surface levels and disrupting LRP6 signalosome formation

We next turned our attention to the mechanism(s) underlying Src/Fer-mediated regulation of Wnt/LRP6 signalling. Since Src leads to a dose-dependent increase of LRP6 protein levels (Fig 2A; Supplementary Fig S1B), we tested whether it alters LRP6 turnover. In HEK293 cells treated with the protein synthesis inhibitor cycloheximide, the half-life of LRP6 is actually increased slightly in the presence of Src (Supplementary Fig S5A). Although somewhat counterintuitive, this is in agreement with a recent study reporting reduced half-life of a LRP6 tyrosine mutant, where increased cell surface levels were also observed [23]. We therefore performed cell surface biotinylation experiments and again in line with the previous report [23], Src leads to a significant reduction in cell surface levels of endogenous LRP6 (Fig 5A). Src overexpression however did not significantly alter cell surface levels of transferrin receptor (TfR), confirming this effect

is specific. It was proposed that altered caveolin-mediated endocytosis of the LRP6 tyrosine mutant was responsible for its altered activity [23], so we next asked whether endocytosis was required for Src-mediated inhibition of Wnt/ β -catenin signalling. Surprisingly, siRNA targeting caveolin and clathrin had no effect on the ability of Src to inhibit Wnt/ β -catenin signalling, although the targeted proteins were significantly reduced (Supplementary Fig S5B).

An important step for LRP6 activation is Wnt-induced LRP6 clustering into activated receptor complexes referred to as LRP6 signalosomes [33]. We investigated the potential effect of Src on endogenous LRP6 signalosome formation using density gradient centrifugation. In HEK293T cells stimulated with Wnt3a, a shift of total LRP6 to heavier fractions (fractions with lower number) is seen, indicating aggregation of LRP6 into larger complexes or aggregates (Fig 5B panel a) and Src blocks this effect (Fig 5B, compare panels a and b). A clearer effect is seen if the Sp1490 antibody is used, which detects the more active PPPSP phosphorylated form of LRP6 (Fig 5B, compare panels c and d). Src inhibits LRP6 signalosome formation in a kinase-dependent manner because only wt but not kd Src shows this effect (Supplementary Fig S5C). Surprisingly, we did not observe any significant effect for Fer on the distribution of LRP6 aggregates (Supplementary Fig S5D). We further investigated the effect of Src on LRP6 signalosome formation in cells of live zebrafish embryos, using fluorescence microscopy-based techniques. In line with our recent report [34], clear LRP6-Wnt positive aggregates can be seen on the plasma membrane of early gastrula cells co-expressing LRP6 and Wnt8a (Fig 5C, panels b–d), indicating the presence of signalosome complexes. Src co-expression strongly disrupts these LRP6-Wnt8a signalosomes (Fig 5C and D), supporting our finding that LRP6 tyrosine phosphorylation by Src prevents the formation or maintenance of active LRP6 signalosomes. Interestingly, although Src efficiently disrupts LRP6 aggregation (Fig 5B, compare panels b and f), membrane-localized clusters of Wnt8 remain intact (Fig 5B, compare panels c and g). Taken together, these data indicate that Src inhibits Wnt/LRP6 signalling by interfering with LRP6 cell surface localization that is accompanied by a destabilization of LRP6 aggregation into active receptor complexes.

Fer-mediated LRP6 inhibition is blocked by CK1 γ

Full activation of LRP6 requires CK1 γ -mediated phosphorylation of LRP6 [11]. In the process of studying the effect of tyrosine phosphorylation events on the Ser/Thr phosphorylation status of LRP6, we made the interesting observation that CK1 γ blocked the ability of Fer to inhibit LRP6-induced Wnt/ β -catenin signalling (Fig 6A). We therefore looked in more detail at the phosphorylation status of LRP6 under different conditions with respect to CK1 γ and Fer activity. In agreement with the TOPFLASH Wnt reporter assay, CK1 γ prevents Fer-induced tyrosine phosphorylation of LRP6 (Fig 6B, p-Tyr, compare lanes 5 and 6). The upshift of LRP6 as well as the induction of the CK1 γ site phosphorylation at T1479 confirms the activity of CK1 γ . The degree of tyrosine phosphorylation of Fer reflects its kinase activity [25] and CK1 γ strongly inhibits this (Fig 5B, pFer, compare lanes 5 and 6). This indicates that CK1 γ inhibits Fer activity, thereby preventing it from phosphorylating LRP6. One prediction would therefore be that CK1 γ loss of function should increase the level of

Figure 7. Proposed model for Src- and Fer-mediated LRP6 regulation.

Inhibition of Fer-mediated LRP6 phosphorylation induced by Wnt-mediated CK1 γ activity (1) (i.e. derepression of LRP6) allows rapid initiation of Wnt-induced LRP6 aggregation (2). Continued Wnt-induced PPPSPxS motif phosphorylation promotes full LRP6 clustering and activity; however, this also induces more tyrosine phosphorylation of LRP6, leading to eventual destabilization of LRP6 aggregates, removal of LRP6 from the cell surface and inhibition of Wnt/ β -catenin signalling via a negative feedback mechanism. The nature of the internalized vesicles that tyrosine-phosphorylated LRP6 is routed to is unclear, but previous studies suggest they are likely to be endocytic vesicles formed after caveolin-mediated endocytosis [23]. Note that Frizzled receptors and other signalosome components are not shown for simplicity.

Fer-mediated LRP6 tyrosine phosphorylation, and this is indeed the case. A kinase dead version of CK1 γ that functions as a dominant negative [11] enhances the ability of Fer to phosphorylate LRP6 (Fig 6B, compare lanes 5 and 7). These results suggest that one of the mechanisms used by CK1 γ to activate LRP6 signalling activity is the de-repression of Fer-mediated LRP6 tyrosine phosphorylation.

Discussion

LRP6 phosphorylation is a central mechanism that couples Wnt/ β -catenin signalling initiation at the membrane to downstream signal transduction [13,35,36]. Here, we report the identification of the non-receptor tyrosine kinases Src and Fer as additional LRP6 regulators. In contrast to the well-documented positive regulation of LRP6 upon Ser/Thr phosphorylation of PPPSPxS motifs, we show that phosphorylation of multiple evolutionarily conserved tyrosine residues by Src and Fer negatively regulates LRP6 signalling. We have demonstrated that both Src and Fer associate with LRP6 and can phosphorylate LRP6 in a direct manner. Gain- and loss-of-function experiments show that this tyrosine phosphorylation of

LRP6 is correlated to an inhibition of Wnt/ β -catenin signalling. Initial *in vivo* analysis using zebrafish embryos supports this and MEF cells derived from mice with genetic ablation of Src as well as from mice with genetic ablation of Fer likewise support a negative regulatory role for these kinases in Wnt/ β -catenin signalling. Based on our results, we propose that initial activation of LRP6 by Wnt is enhanced by CK1 γ -dependent repression of Fer-mediated LRP6 phosphorylation (LRP6 derepression mechanism) and that continued Wnt signalling and LRP6 receptor activation is kept in check by a negative feedback mechanism that disrupts signalosomes and removes LRP6 from the cell surface (Fig 7). Although our results for siRNA silencing of caveolin and clathrin are not in line with the proposed role of endocytosis recently reported to be linked to LRP6 tyrosine motifs [23], our analysis on cell surface LRP6 levels are. It is therefore likely that tyrosine phosphorylation of LRP6 interferes with both LRP6 signalosome formation and endocytic routing of LRP6 away from the cell surface. Differences in experimental conditions may influence which of these processes is preferentially affected in different studies. Interestingly, a recent study has suggested that endocytic regulation of Wg signalling takes place downstream of activated receptor complexes [37]. Thus, although caveolin-mediated endocytosis is clearly implicated in LRP6

functionality [23,38,39], there is some debate as to how endocytosis functions to regulate Wnt signalling and it will be important to address this in future studies.

Tyrosine phosphorylation of LRP6 by Src and Fer

Our data demonstrate that Src phosphorylates LRP6 in a Wnt-dependent manner, which for the first time provides a direct biochemical link between tyrosine phosphorylation of a Wnt receptor to Wnt signalling regulation. Src is a multifunctional kinase that has been well studied for decades and it comes as a surprise that it should regulate LRP6 in such a direct manner. Src, Yes and Fyn constitute the functionally redundant, ubiquitously expressed Src family kinase members and it is possible that all function as LRP6 kinases, although only Src was identified in our screen. A more detailed analysis of their specific effects on LRP6 will be worth exploring. Likewise, whether Fes, which is highly related to and partly redundant with Fer, also functions to regulate LRP6 should be investigated.

Src can phosphorylate both LRP6 protein bands that are visible after SDS-PAGE and Western blot analysis using a total LRP6 antibody (see e.g. Fig 1D). Either Src has the ability to phosphorylate immature LRP6, which corresponds to the lower band that is less abundantly glycosylated, or Src phosphorylation of LRP6 promotes cell surface LRP6 deglycosylation and/or proteolysis events. We have tested the effect of Src on an ER retained LRP6 mutant [40] and this indeed indicates that Src can phosphorylate and inhibit ER-localized LRP6 (Y.S., data not shown). Src is known to regulate and be regulated by trafficking processes [41,42], and we cannot exclude that its overexpression could potentially result in partial retention of LRP6 in the ER/Golgi compartment. Nevertheless, Fer also inhibits Wnt/ β -catenin signalling upon phosphorylation of LRP6 and this is correlated almost exclusively with an increased tyrosine phosphorylation of the upper, cell surface, LRP6 band. We also see reduction of cell surface LRP6 protein levels by Src, which, taking into account the increase in total protein levels, suggests that Src promotes either internalization or ER/Golgi retention of LRP6. The fact that overexpression of Src does not influence transferrin receptor levels at the cell surface (Fig 5A) implies a specific function of Src on LRP6 routing away from the cell surface.

Multiple tyrosine residues contribute to Src-mediated LRP6 phosphorylation and they likely function in a cooperative manner to inhibit LRP6 signalling, much like the cooperatively seen between the PPPSPxS motifs [43]. This is supported by the combined mutation of the four most relevant sites, which strongly reduces both LRP6 tyrosine phosphorylation and its inhibition by Src. It will nevertheless be interesting to investigate their individual behavior in detail. Interestingly, compared to full-length LRP6, the constitutively active LRP6- Δ E1-4 is sensitized to Src-mediated tyrosine phosphorylation (Fig 3A, compare wt and Δ E1-4 wt), suggesting the activation state of LRP6 regulates its phosphorylation at tyrosine residues. This phenomenon has been observed for both CK1 and PPPSP site phosphorylation of LRP6 [11] and is also in line with our proposed negative feedback mechanism for LRP6 tyrosine phosphorylation.

Mechanism of Fer-mediated LRP6 inhibition

It appears that Src and Fer use distinct mechanisms to regulate the function of LRP6 in Wnt/ β -catenin signalling (Fig 7). Interestingly,

Fer-mediated LRP6 tyrosine phosphorylation is blocked by CK1 γ , providing an additional mechanism of how CK1 γ activates LRP6. We propose that this de-repression of LRP6 by CK1 γ allows a faster, more robust initiation of Wnt-stimulated signalosome formation (Fig 7). Basal as well as higher Wnt-induced levels of tyrosine phosphorylation can be detected on endogenous LRP6, which is a prerequisite for the proposed CK1 γ de-repression model. It remains to be established exactly how Fer-mediated tyrosine phosphorylation inhibits LRP6 signalling, considering it does not appear to function in the same way as Src to disrupt LRP6 aggregation. Although, in our hands, we could not confirm that LRP6 tyrosine phosphorylation plays a significant role in regulating the endocytosis of LRP6, there is recent evidence suggesting endocytosis of LRP6 is regulated by tyrosine-based motifs [11,23]. It is also interesting to note that RAB8B was recently identified as a regulator of LRP6 endocytosis and that it also interacts with CK1 γ and has been proposed to regulate signalosome formation [38]. It will also be interesting to look in more detail at the role of Fer-mediated LRP6 phosphorylation on segregation of LRP6 between lipid-raft and non-lipid-raft membrane compartments, which have been reported to have distinct functions in trafficking LRP6 and CK1 γ as well as receptor activation and internalization [44,45]. Clearly, further studies will be necessary to clarify the precise details of how LRP6 activity is regulated by the tyrosine phosphorylation events reported here.

Mechanism of Src-mediated LRP6 inhibition

Src-mediated LRP6 phosphorylation appears to inhibit LRP6 function by disrupting LRP6 aggregation and we therefore propose that Src prevents signalosome formation and/or maintenance at the cell surface (Fig 7). The fact that Wnt can increase the level of LRP6 tyrosine phosphorylation also indicates that it operates as a negative feedback mechanism to prevent overactivation of the pathway. A recent study has shown that mutation of a YxxYxYxx Φ tyrosine-based motif in the LRP6 ICD (YRPYSYRHF) results in increased cell surface LRP6 levels and decreased rates of endocytosis [23]. This motif includes one of the 4 completely conserved tyrosine residues that we show is phosphorylated by Src and contributes to Src-mediated inhibition of LRP6 signalling (Y1520). A previous study also hinted at a potential role in endocytic regulation of LRP6 via the same tyrosine-based motif. This was based on the claim that it mediates association with the endocytic adaptor protein, AP-2 [22]. Although, as mentioned above, we cannot rule out the possibility that endocytosis plays a part in the inhibition of LRP6 function upon tyrosine phosphorylation by Src and Fer, our data point to alternative mechanisms involving receptor aggregation. Only the centrally positioned Y1520 tyrosine residue within the YxxYxYxx Φ endocytic motif was confirmed to be phosphorylated by our MS studies, raising the question of whether Src- and Fer-mediated phosphorylation of LRP6 is distinct from the functionality of this motif. Future studies will be required to establish which mechanism(s) operate under particular cellular contexts and it is likely that a combination of LRP6 aggregation events, Ser/Thr phosphorylation events and endocytosis events are involved. Both Src- and Fer-mediated LRP6 inhibitory mechanisms should operate simultaneously as both kinases are ubiquitously expressed; however, the relative degree to which they repress LRP6 is likely to be context dependent.

In vivo relevance

Wnt/ β -catenin signalling regulates vertebrate anteroposterior (a-p) patterning [31]. In zebrafish, this is best seen through a reduction in the size of the eyes in response to Wnt/ β -catenin signalling gain of function [46,47]. Our data show that Src mRNA injection in zebrafish embryos results not only in reduced expression of the Wnt/ β -catenin target gene *Axin2*, but also rescues the small eye phenotype caused by LRP6 mRNA injection. Considering Src null mice develop osteopetrosis [26] and LRP5/6 has been implicated in the regulation of bone mass by regulating the function of osteoblasts [48,49], it is tempting to think there may be a connection.

Cross talk between Src/Fer and Wnt signalling

There are several reports of cross talk between Src and Wnt signalling. Src can phosphorylate β -catenin and promote its release from adhesion complexes [50] and this has been linked to activation of Wnt signalling [51,52]. However, other studies conclude that tyrosine phosphorylation does not affect β -catenin abundance and/or its transcription activity [53–55]. Overexpression of Src and Fer as well as activation of the receptor tyrosine kinases EGFR and c-Met all downregulate E-cadherin-mediated adhesion and have been correlated with an increase in β -catenin tyrosine phosphorylation [56]. It has also been reported that Src docks to and becomes activated by Dvl2 in a Wnt-dependent manner [28] and activated Src was reported to phosphorylate β -catenin and Dvl2 to promote Wnt signalling [28]. Although this Dvl docking model may explain one way of how Src can gain access to LRP6, our data show that Src has a negative, not positive, effect on Wnt/ β -catenin signalling. In *C. elegans*, Src is reported to be an important regulator of Wnt signalling [29,57]. Although no homolog of LRP6 has been identified in *C. elegans*, it has been postulated that additional substrates are likely targeted by Src to account for its effects in Wnt signalling. Interestingly, both Src and Fer have been suggested to target the *C. elegans* β -catenin homolog HMP-2; however, Src in a positive manner and Fer in a negative manner [29,30].

Supporting our GoF data which indicate that Src and Fer phosphorylate LRP6 to inhibit its signalling function in the Wnt/ β -catenin pathway, Src (SYF) knockout cells show that a loss of LRP6 tyrosine phosphorylation is associated with a markedly increased responsiveness to Wnt3a stimulation. The fact that this can be rescued by reintroduction of Src alone suggests that Src is both necessary and sufficient for repressing Wnt/ β -catenin signalling. Nevertheless, Src, like GSK3 [58], is a multifunctional kinase in Wnt signalling and likely can function either in a positive or negative regulator manner depending on cellular context.

Materials and Methods

Antibodies, plasmids and siRNA

Rabbit polyclonal anti-Yp1460 (LRP6) antibody was raised after injection of rabbits with a synthetic phospho-peptide (NH₂-GGSSGPPYpDRAHVTGAS-COOH) and subsequently immunopurified using these and the corresponding non-phosphorylated peptides. Rabbit polyclonal anti-T1479, Sp1490 and Tp1479

antibodies have been described previously [11]. Other antibodies used were as follows: rabbit polyclonal anti-Sp1490, mouse monoclonal anti-Fer, mouse monoclonal anti-pan-phospho-tyrosine, rabbit monoclonal anti-caveolin-1 (Cell Signalling); rabbit polyclonal anti-Src, mouse monoclonal anti- α -tubulin (Santa Cruz); mouse monoclonal anti-Flag, anti-Flag M2 Agarose beads (Sigma); mouse monoclonal anti- β -actin (Abcam); mouse monoclonal anti-Myc, mouse monoclonal anti-V5 (hybridoma) mouse monoclonal anti-clathrin heavy chain (BD bioscience).

LRP6 Y-F mutants and SrcK298M kinase dead constructs were generated by PCR using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Human LRP6-mCherry was generated by replacing the EGFP open reading frame (ORF) of pCS2+ hLRP6-EGFP [59] with the ORF of mCherry, between XbaI and SnaBI sites. Other constructs have been described previously [10,11,34]. TOPFLASH constructs have been previously described [60]. Control siRNA (siGENOME Non-Targeting Control siRNA) and siRNA against human LRP6 (siGENOME human LRP6 siRNA SMARTpool) were purchased from Dharmacon. siRNA against human clathrin heavy chain (CHC) and caveolin-1 were previously described [39].

Medaka cDNA screen for LRP6 tyrosine kinase

A Medaka cDNA library was used as a source of potential LRP6 kinases [61]. Blast analysis of the annotated sequence data from this library was used to identify all clones harboring kinase domains and these clones were picked from the master library, amplified and arrayed in 96-well plates. A total of 240 unique kinases were identified and arrayed. Transfection ready pDNA was prepared for all clones. HEK293T cells in 96-well plate were transfected with 40 ng pCS2+-6×Myc-human-LRP6 and 5 ng of the Medaka kinase clones using Promofectin (Promokine) according to manufacturer's instructions. After one day, cells were lysed in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.0], 150 mM NaCl, 25 mM NaF, 5 mM Na₃VO₄ and protease inhibitor cocktail [Roche]) and harvested for SDS-PAGE/Western blot analysis to detect total LRP6 and LRP6 tyrosine phosphorylation levels with anti-Myc and anti-pan-phospho-tyrosine antibodies, respectively. Overexpression of LRP6 allowed LRP6 tyrosine phosphorylation to be detected by the pan-phospho-tyrosine Antibody (panYp) at a sensitivity significantly above that required to detect any tyrosine phosphorylation of endogenous cellular proteins.

Cell culture, cell transfection, Western blot, luciferase reporter assay

All cells were maintained at 37°C and 5% CO₂ in DMEM containing 10% FCS. Wnt3a-conditioned medium was produced from mouse L cells stably transfected with mouse Wnt3a- and control-conditioned medium was from non-transfected L cells (ATCC CRL-2647 and CRL-2648, respectively).

HEK293T cells were transfected with plasmid DNA using either Promofectin (Promokine) or ScreenFectA (Incella), and MEF cells were transfected with plasmid DNA using Attactene (Qiagen). For siRNA transfection in HEK293T cells, ScreenFectA (Incella) was used. For TOPFLASH, luciferase Wnt reporter assays cells were harvested 24 h post-transfection in passive lysis buffer (Promega). For Western blot analysis, cells were harvested in 1% Triton lysis

buffer. All error bars shown are standard deviation (SD) from mean of triplicates. Student's *t*-test was performed as a homoscedastic test with two-tailed distribution to determine the significance (**P* < 0.05, ***P* < 0.01).

In vitro kinase assay

pCS2 + -Flag-human LRP6, *pCS2* + -Flag-human Src or *pCS2* + -Flag-Medaka-Fer were transfected separately in HEK293T cells in 10-cm plates. After one day, cells were lysed in 1 ml 1% Triton lysis buffer and LRP6, Src or Fer immunoprecipitated overnight at 4°C using anti-Flag M2 beads (Sigma). Beads were washed five times with 1% Triton lysis buffer containing 0.3 M NaCl and equilibrated in kinase buffer lacking ATP. Reactions were started by combining 10 µl packed bead volume of LRP6 and Src and supplemented with 6 µl 1 mM ATP (100 µM end-concentration) and incubated for 1 h at 30°C. As control reaction, 30 µl beads slurry of LRP6 alone was instead mixed with 30 µl kinase buffer. The reaction was stopped by adding 20 µl 4× Laemmli buffer, and the beads were eluted by heating at 95°C for 5 min. Supernatant was then used for SDS-PAGE/WB analysis.

Membrane protein fractionation

MEF cells in 6 well were detached with low salt buffer (5 mM HEPES [pH 7.0], 1 mM MgCl₂, 10 mM NaF, 5 mM Na₃VO₄ and protease inhibitor cocktail) and homogenized using a Dounce homogenizer (40 strokes). Lysates were spun at 500 g for 5 min to remove nuclei, followed by a spin at 10,000 g for 10 min to pellet the membrane fraction. The membrane pellets were dissolved in 1% Triton lysis buffer and subjected to Western blot analysis.

Cycloheximide (CHX) assay for LRP6 half-life study

HEK293T cells overexpressing either hLRP6 (control) or hLRP6 + hSrc were treated with cycloheximide (CHX, 20 µg/µl) for different times (0, 3, 6, 9 h), and LRP6 protein was assessed by Western blot and normalized to actin expression. Densitometry analysis was performed using Image Studio software (Li-COR). All values were normalized to time point 0 (set as 1).

Cell surface protein labeling with biotin

Biotin labeling of cell surface protein was performed at 4°C using 0.5 mg/ml EZ-Link-Sulfo-NHS-SS-Biotin (Pierce). Cell proteins were extracted with 1% Triton X-100 lysis buffer and biotinylated proteins precipitated with immobilized NeutrAvidin beads (Pierce) according to manufacturer's instruction. Total and cell surface biotin-labeled proteins were subjected Western blot analysis as described above (WB).

Opti-Prep density gradient centrifugation

One day post-transfection, HEK293T cells in 10-cm plates were treated for 3 h with Wnt3a- or control-conditioned medium before harvest. Cells (in two 10-cm plates) were harvested in Hank's Balanced Salt buffer on ice, pelleted and then lysed for 20 min on ice in 550 µl of extraction buffer (1% Triton X-100, 30 mM Tris-HCl [pH 7.4], 25 mM NaCl, 3 mM Na₃VO₄ and protease

inhibitor cocktail). Lysates were centrifuged, and 50 µl supernatant of each sample was taken as input, and the remaining 500 µl sample was layered carefully on top of 4.5 ml of 15–40% Opti-Prep (Sigma) gradient. Ultracentrifugation was performed in a Beckman SW50.1 rotor at 45,000 g for 4 h at 4°C. After centrifugation, 12 fractions of 400 µl were collected and analyzed by SDS-PAGE/WB.

Zebrafish manipulation, mRNA injection, in situ hybridization

All zebrafish husbandry and experimental procedures were performed in accordance with the German law on Animal Protection and were approved by Local Animal-Protection Committee (Regierungspräsidium Karlsruhe, Az.35-9185.64) and the Karlsruhe Institute of Technology (KIT). The data we present in this study were acquired from analysis of KIT wild-type zebrafish AB202. Breeding zebrafish (*Danio rerio*) were maintained at 28°C on a 14 h light/10 h dark cycle [62]. mRNA of human LRP6, Medaka Src and mCherry as control were injected at one-cell stage. *In situ* hybridization with anti-Axin2 probe was performed 24 h post-fertilization as described [63].

Microscopy

For ISH and phenotype analysis, embryos were imbedded in 70% glycerol/PBS (v/v). Images were taken on Olympus SZX16 microscope equipped with a DP71 digital camera by using Cell A imaging software. For confocal analysis, live embryos were embedded in 0.7% low melting agarose (Sigma-Aldrich) dissolved in 1× Ringer's solution. Images were obtained with Leica TCS SP5 X confocal laser-scanning microscope using 40× or 63× dip-in objectives. Image processing was performed with Imaris 6.3.1 software (Bitplane AG). For confocal analysis of signalosomes, images of live embryo at 30–50% epiboly were quantified for the colocalization of Lrp6 and Wnt8 by the Mander's colocalization coefficient [34].

Mass spectrometry analysis

After detachment with Accutase, freshly resuspended HEK293T cells were transfected with 50 µg Flag-LRP6 with or without 15 µg hSrc using ScreenFectA (Incella GmbH) using the one-step transfection protocol and plated directly in 175-cm² flasks. Flag LRP6 proteins were purified from lysates 48 h post-transfection using anti-Flag M2 beads (Sigma) and separated using 7.5% SDS-PAGE gels. Bands corresponding to the LRP6 protein were excised from the gel and subjected to Tryptic digestion followed by LC-MS-MS analysis using an Orbitrap Elite (Thermo) to measure the peptides (ZMBH Mass Spectrometry facility, Heidelberg). For the identification of the phosphorylation site, the open-source search algorithm MaxQuant was used and for the quantification shown in Fig 3B; Supplementary Fig S3B, the summed up peak areas from extracted ion chromatograms were used.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

QC and YS performed the majority of experiments for Src and Fer, respectively. JWe prepared samples for and organized the MS analysis. AIH and QC planned and performed the zebrafish experiments with supervision from SS. MR performed the bioinformatics analysis to identify kinases in the cDNA library and JWi provided the Medaka cDNA master library. QC, YS and GD planned experiments and prepared figures. GD supervised the project and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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