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Nontransformed cells form heterotypic cadherin junctions with adjacent transformed cells to inhibit tumor cell growth and motility. Transformed cells must override this form of growth control, called "contact normalization", to invade and metastasize during cancer progression. Heterocellular cadherin junctions between transformed and nontransformed cells are needed for this process. However, specific mechanisms downstream of cadherin signaling have not been clearly elucidated. Here, we utilized a β-catenin reporter construct to determine if contact normalization affects Wnt signaling in transformed cells. β-catenin driven GFP expression in Src transformed mouse embryonic cells was decreased when cultured with cadherin competent nontransformed cells compared to transformed cells cultured with themselves, but not when cultured with cadherin deficient nontransformed cells. We also utilized a layered culture system to investigate the effects of oncogenic transformation and contact normalization on gene expression and oncogenic Src kinase mediated phosphorylation events. RNA-Seq analysis found that cadherin dependent contact normalization inhibited the expression of 22 transcripts that were induced by Src transformation, and increased the expression of 78 transcripts that were suppressed by Src transformation. Phosphoproteomic analysis of cells expressing a temperature sensitive Src kinase construct found that contact normalization decreased phosphorylation of 10 proteins on tyrosine residues that were phosphorylated within 1 h of Src kinase activation in transformed cells. Taken together, these results indicate that cadherin dependent contact normalization inhibits Wnt signaling to regulate oncogenic kinase activity and gene expression, particularly PDPN expression, in transformed cells in order to control tumor progression.

Keywords Src kinase, PDPN, Podoplanin, Contact normalization, Cell transformation

Over 19 million new cancer cases are diagnosed each year. In spite of significant strides in research, the overall 5 year cancer survival rate has remained at about 50% for the past several years. Accordingly, cancer killed over 600,000 people in the United States and over 9 million globally in 2020 [1,2]. There is a clear need to better understand molecular pathways that drive cancer progression.

Ultimately, cancer is regulated by oncogenes and tumor suppressers. The Src tyrosine kinase is a powerful oncogenic that phosphorylates effectors to increase nonanchored tumor cell growth and motility required for invasion and metastasis [3–5]. Src is not mutated in most cancers. However, Src activity is associated with many types of cancer, including tumors of the colon, breast, pancreas, brain, and squamous cell carcinoma [6–8]. Src is an enticing target for chemotherapy, but its activity is too ubiquitous for specific targeting [9–11].

Src phosphorylates the adaptor protein Cas to increase podoplanin (PDPN) expression in order to promote tumor cell motility, invasion, and metastasis. Src transformed cells do not form invasive tumors in the absence

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of Cas or PDPN [12–14]. PDPN is a transmembrane receptor that promotes tumor cell motility, invasion, and metastasis. PDPN regulates the activities of Rho, ezrin, and other proteins linked to the actin cytoskeleton to mediate filopodia formation, cell motility, invasion, and metastasis. Indeed, PDPN expression enhances the motility and invasion of several neoplastic cell types including mammary carcinoma, glioma, melanoma, and squamous cell carcinoma [4,15–18].

However, cancer progression is a dynamic process. Nontransformed cells can normalize the growth and morphology of adjacent transformed cells. This process is called "contact normalization" and can control tumor cell growth and expansion. Transformed cells must overcome contact normalization in order to become invasive and metastatic. This form of heterocellular growth control can direct contact inhibition of locomotion and proliferation [19–24].

Contact normalization is a powerful process. Genetically transformed cells can assume a normal morphology and reside in many organs including breast, intestine, and skin. Cells transformed by a variety of chemicals, viral agents, and oncogenes including the Src kinase, can be normalized by contact with nontransformed cells [21–24].

Contact normalization requires direct junctional communication between transformed and nontransformed cells. Cellular communication by diffusible factors is not sufficient to mediate this process [22,25]. Nontransformed cells must establish heterocellular cadherin junctions with neighboring transformed cells to normalize their growth and morphology [21]. However, mechanisms by which cadherins mediate this process have not yet been elucidated.

Cadherins form intercellular junctions that are required to maintain normal cell architecture. Cadherins are tethered by catenins to the actin cytoskeleton. Src can phosphorylate cadherins and β-catenin to disrupt these junctions. In addition to disturbing cell morphology, disruption of cadherin junctions allows β-catenin to enter the nucleus and drive oncogenic gene expression [26,27]. Commonly expressed "classical" cadherins include E-cadherin (E-Cdh) and N-cadherin (N-Cdh). Aberrant expression of these cadherins is associated with increased Wnt/β-catenin signaling and cancer progression [28,29].

Here, we performed comprehensive mRNA and phosphoproteomic analyses to identify genes and proteins affected by cadherin mediated contact normalization of Src transformed cells. We also utilized a β-catenin driven reporter construct to find that contact normalization inhibits Wnt signaling in these transformed cells. In particular, these data implicate the PDPN receptor, lymphocyte antigen 6 family member C1 (LY6C1), aspartoacylase (ASPA), four and a half LIM domains 1 (FHL1), and annexin A2 (ANXA2) as key proteins inversely affected by oncogenic Src kinase activity and cadherin mediated contact normalization.

Experimental procedures

Cell culture

Nontransformed mouse embryonic cells (Mec), vSrc transformed mouse embryonic cells (MecSrc), nontransformed homozygous null N-Cdh knockout mouse embryonic cells (MecCdhKo), vSrc transformed mouse embryonic cells with constitutive PDPN expression (MecSrcPdpn), nontransformed homozygous null PDPN knockout cells (PdpnKo), nontransformed mouse embryonic cells with constitutive PDPN expression (PdpnWt), temperature sensitive Src (tsSrc) transformed homozygous null PDPN knockout (tsSrcPdpnKo) cells, and temperature sensitive Src (tsSrc) transformed mouse embryonic cells with constitutive PDPN expression (tsSrcPdpnWt) have been previously described [21,30]. Nontransformed (Mec) and vSrc transformed (MecSrc) mouse embryonic cells were transfected with a Wnt signaling reporter construct designed with EGFP expression driven by a minimal TA viral promoter enhanced by 7 copies of a functional (Genscript #U571DGK100_5) or nonfunctional (Genscript #U571DGK100_9) TCF/LEF binding site, and a hygromycin phosphotransferase cassette for selection. These nontransformed (TopGfpMec) and Src transformed (TopGfpMecSrc) cells express a β-catenin inducible EGFP, or noninducible EGFP (FopGfpMec and FopGfpMecSrc cells) as described for β-catenin inducible M50Super8xTopFlash and noninducible M51Super8xFOPFlash luciferase expression [31–33]. Cells were cultured with or without 10 mM LiCl (Thermo Scientific #449041000) or 10 μM IWP-2 (Selleck Chemical #S7085) for 48 h or 10 h in DMEM (Mediatech, inc., 10–014-CV) supplemented with 25 mm HEPES (Mediatech, inc., 25-060-Cl), and 10% FBS (Serum Source International, Inc., DH5293) at 37 °C, 5% CO_2, and 100% humidity as previously described [21,30]. Cells expressing tsSrc were cultured at the nonpermissive (39 °C) temperature or permissive (34 °C) temperatures to repress or induce Src activity, respectively, as described [30]. For some experiments, transformed cells were stained with DiD (AAT Bioquest #22034) before plating to identify them in cocultures with other cells at a 1:5000 ratio, cultured with or without 10 mM LiCl for 48 h, and visualized by phase contrast and fluorescent microscopy with a Zeiss Axiovert 5 fluorescence, or with a Zeiss Axiovert 40 CFL microscope equipped with filter sets to detect GFP (excitation, 470 ± 40; emission, 525 ± 50) and DiD (excitation, 631 ± 33; emission, 709 ± 100) fluorescence equipped with a ZeissAxioCam Mrc camera and Zen software as previously described [21,30].

For some experiments, a layered culture system was used to allow separated populations of transformed and nontransformed cells to form intercellular junctions with each other. Briefly, 100,000 vSrc transformed (MecSrc), PDPN transfected v-Src transformed (MecSrcPdpn), nontransformed cadherin competent (Mec), or nontransformed cadherin deficient (MecCdhKo) cells were plated on porous membranes (Costar 3542) containing 300,000 vSrc transformed (MecSrc), PDPN transfected v-Src transformed (MecSrcPdpn), nontransformed cadherin competent (Mec), or nontransformed cadherin deficient (MecCdhKo) cells on the other side. Cells on the top and bottom of the membrane form intercellular junctions with each other through the 3 micron pores in the membrane which prevent cells from migrating to the other side. Cells were harvested and analyzed 24 h after plating as described [21,25,34–36].

Western blot analysis

Western blotting was performed as previously described [21,30]. Briefly, protein from cells lysed in lysis buffer (2% SDS, 10% glycerol, 50 mM DTT in 62.5 mM Tris–HCl, pH 6.8) was resolved by 10% SDS-PAGE (18ug/lane), transferred to Immobilon-P membranes (EMD Millipore #IPVH00010), and incubated with antisera specific for mouse PDPN (University of Iowa Developmental Studies Hybridoma Bank #8.1.1), GAPDH (Santa Cruz #FL335), β-actin (Sigma #A1978), Pan-Cdh (Sigma #C1821), N-Cdh (BD Biosciences #610920), active Src kinase phosphorylated at Tyr 416 (Cell Signaling Technology #2101), ASPA (Abcam #AB223269), Ly6c1 (Cell Signaling #3787), Fhl1 (Proteintech #S1009), Anxa2 (Proteintech #66035-1-IG), Anxa2 phosphorylated at Tyr 238 (Invitrogen # PA5105372), EIF3C (Cell Signaling #2068), Ddx3 (Cell Signaling #8192), Parp12 (Invitrogen # PIPA513311), Cttn (Invitrogen #PIMA515831), Sec31a (Invitrogen # PIMA531900), PALM2/AKAP2 (Invitrogen # PIPA5140342), Aqp5 (Proteintech #20334-1-AP and Abcam #AB305303), Acly (Proteintech #67166-1-IG), Tmem45a (Abcam #AB166899), and Dctn3 (Abcam #AB124674). Primary antiserum was recognized by appropriate secondary antiserum specific for mouse (Invitrogen #31430), rabbit (Santa Cruz #sc-2305), rat (Sigma Millipore #AP136P), or Syrian hamster (Santa Cruz #sc-2493) IgG conjugated to horseradish peroxidase and detected by enhanced chemiluminescence (Thermo Scientific #32209). Membranes were stained with India ink and protein gels were stained with Coomassie blue to verify equal loading and transfer.

RNA-Seq analysis

Nontransformed (Mec) cells, nontransformed cadherin deficient (MecCdhKo) cells, vSrc transformed (MecSrc) cells with endogenous PDPN expression, and PDPN transfected vSrc transformed (MecSrcPdpn) cells were grown with themselves and each other in the layered culture system for 24 h. RNA was extracted and sequenced as described [21]. Sample reads were counted with featureCounts (version 1.6) [37] and analyzed for differences in expression with DESeq2 (version 1.26.0) [38] by R (R Foundation for Statistical Computing, Vienna, Austria, version 3.6.3) with p-values calculated by the Wald test and p-adjusted values calculated by the Benjamini–Hochberg method. RNA sequencing data have been deposited into the NCBI sequence read archive (SRA) database under the accession number PRJNA1070015 at https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1070015.

Phosphopeptide analysis

Temperature sensitive (tsSrc) transformed PDPN competent (tsSrcPdpnWt) cells and tsSrc transformed PDPN deficient (tsSrcPdpnKo) cells were grown in standard culture for 24 h at the nonpermissive temperature (39 °C) followed by 1 h at permissive temperature (34 °C). Nontransformed (Mec) cells, nontransformed cadherin deficient (MecCdhKo) cells, vSrc transformed (MecSrc) cells with endogenous PDPN expression, and PDPN transfected vSrc transformed (MecSrcPdpn) cells were grown with themselves and each other in the layered culture system for 24 h. Protein was extracted from these cells and analyzed by phosphopeptide mass spectrometry as described [30]. Briefly, protein was quantitated, reduced with DTT and iodoacetamide, and digested with trypsin. Peptides were labeled with Thermo TMTpro, fractionated by high pH RPLC, solubilized in ammonium hydroxide, resolved by C18 HPLC, enriched for phosphorylated peptides by IMAC, and analyzed by (LCMSMS) with an Orbitrap Eclipse Tribrid Mass Spectrometer equipped with an Acclaim PepMap 100 trap column (Thermo Fisher) in line with a nano analytical column (nanoEase, MZ peptide BEH C18, Waters). The DDA method was used for analysis of phosphorylated peptides as described [30]. Data can be accessed as MassIVE data set number MSV00009421 at ftp://massive.ucsd.edu/v07/MSV000094121.

Statistical analysis

Sequential binary comparisons were performed as a filtration strategy to find differentially expressed genes and protein phosphorylation sites. Genes affected by oncogenic Src kinase were found with a change of at least 400% between nontransformed (Mec) and vSrc transformed (MecSrc) cells; genes affected by direct heterocellular communication were found with a change of at least 50% between MecSrc cells cultured with themselves or nontransformed (Mec) cells; and genes affected by cadherin dependent contact normalization were found with a change of at least 100% between (MecSrc) cultured with cadherin competent nontransformed (Mec) cells, but not significantly affected by contact with cadherin deficient (MecCdhKo) cells. Phosphorylation events affected by Src kinase were found with a change of least 500% in transformed (MecSrc) cells compared to nontransformed (Mec) cells; events affected by heterocellular contact were found with a change of at least 300% in transformed (MecSrc) cells cultured with themselves compared to with nontransformed (Mec) cells; events affected by cadherin dependent contact normalization were found with a change of at least 200% in MecSrc cells cultured with cadherin competent nontransformed (Mec) cells compared to with cadherin deficient nontransformed (MecCdhKo); and early Src dependent events were found with a change of at least 50% greater within 1 h of induction of Src kinase activity at the permissive temperature in cells expressing temperature sensitive Src (tsSrcPdpnWt) compared to the nonpermissive temperature. Two-tailed Student's t-test was used to identify differences between values. Number of repeats and p values are presented in results and figure legends describing each experiment. Excel (Microsoft) and Prism (Graphpad) software were used for analyses.

Results

Contact normalization affects mRNA expression in Src transformed cells

We employed a panel of established cell lines for this study that is well suited to investigate the effects of oncogenic transformation and contact normalization on gene expression. vSrc transformed (MecSrc) cells, cadherin deficient nontransformed (MecCdhKo) cells, and vSrc transformed cells with forced PDPN expression (MecSrcPdpn) were generated from parental mouse embryonic (Mec) cells. These cells are well characterized. They exhibit physiologically relevant gene expression profiles and robust contact normalization as previously

described [21,25,30,34–36]. Appropriate Src kinase activity and cadherin expression was confirmed in these cells by Western blot analysis as shown in Fig. 1a.

Nontransformed and vSrc transformed cadherin competent and deficient cells were grown in a layered culture system to analyze the effects of contact normalization on gene expression. This system separates cells on each side of a porous membrane that allows intercellular junction formation and communication as shown in Fig. 1b. Cells form intercellular junctions with each other within a few hours after plating them together in this system [39–42]. However, the membrane does not allow mixing of the cells and maintains cell populations that can be efficiently harvested for analysis [21,25,34–36,40,42,43].

Cells were cultured with themselves or each other in the layered culture system for 24 h and their mRNA was analyzed by RNA-Seq as described [21]. A total of 55,376 transcripts was detected in either nontransformed (Mec, MecCdhKo) or vSrc transformed (MecSrc, MecSrcPdpn) cells as shown in Fig. 2a. Oncogenic Src kinase activity affected the expression of 3742 of these transcripts (6.8% of the transcriptome) in these cells. These transcripts were differentially expressed with a fold change of at least 400% ($p < 0.05$ by t-test with $n = 3$) in nontransformed (Mec) cells compared to vSrc transformed (MecSrc) cells. Src induced 2702 (72%) and suppressed 1040 (28%) of these transcripts, with Src induced and suppressed transcripts considered potential tumor promoters and suppressers, respectively, as shown in Fig. 2a,b.

Direct heterocellular junctional communication between transformed and nontransformed cells is needed for contact normalization [4,21,22,25,35,36]. Contact normalization affected the expression of 555 (14.8%) of the 3742 transcripts affected by Src kinase activity with a fold change of at least 50% ($p < 0.05$ by t-test with $n = 3$). vSrc transformed (MecSrc) cells cultured with themselves expressed 271 of these transcripts at least 50% more, and 284 of these transcripts at least 50% less than Src transformed (MecSrc) cells cultured with nontransformed (Mec) cells, with Src induced and suppressed transcripts considered potential tumor promoters and suppressers, respectively, as shown in Fig. 2b,c.

Nontransformed cells form heterocellular cadherin junctions with adjacent transformed cells to normalize their growth and morphology [21]. The expression of 100 of the transcripts affected by contact normalization relied on cadherin expression in nontransformed cells. The expression of these genes in vSrc transformed (MecSrc) cells was affected by at least 100% by contact with cadherin competent nontransformed (Mec) cells, but not significantly affected by contact with cadherin deficient (MecCdhKo) cells ($p < 0.05$ by t-test with $n = 3$). The expression of 22 of these transcripts was decreased, while the expression of 78 were increased by contact with nontransformed cells, with Src induced and suppressed transcripts considered potential tumor promoters and suppressers, respectively, as shown in Fig. 2c,d.

PDPN expression enables transformed cells to override contact normalization [21]. We therefore sought to identify genes with expression consistent with this activity. PDPN transfected vSrc transformed (MecSrcPdpn) cells were used to investigate how PDPN expression affected the expression of potential contact normalization effectors. Forced PDPN expression affected the expression of 32 transcripts associated with cadherin dependent contact normalization. The expression of 6 of these transcripts was increased in vSrc transformed cells expressing exogenous PDPN (MecSrcPdpn) compared to Src transformed cells without forced PDPN expression (MecSrc), while the expression of 26 transcripts was suppressed in this comparison as shown in Fig. 2d,e. Relative expression of these transcripts in nontransformed (Mec), cadherin deficient (MecCdhKo), Src transformed (MecSrc), and

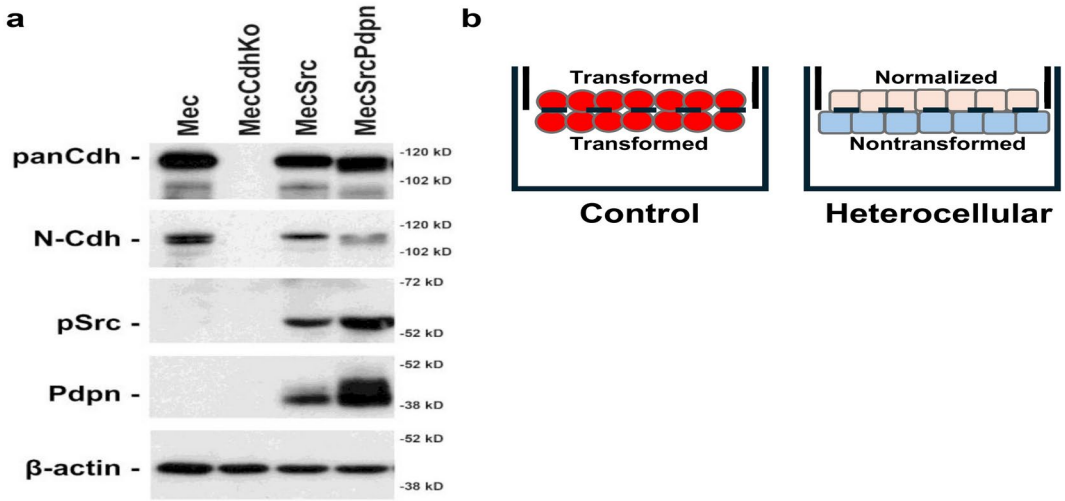


Fig. 1. Cadherin, Src kinase, and PDPN expression in transformed and nontransformed cells. (**a**) Pan-Cdh, N-Cdh, active Src (phosphorylated at Tyr 416), PDPN, and β-actin, were detected by Western blot analysis of protein from nontransformed cadherin competent (Mec) cells, nontransformed cadherin knockout (MecCdhKo) cells, vSrc transformed (MecSrc) cells, and PDPN transfected vSrc transformed (MecSrcPdpn) cells as indicated. (**b**) Transformed cells form junctions with themselves or nontransformed cells through a porous membrane in a layered culture system that maintains them as populations that can be quickly separated and analyzed.

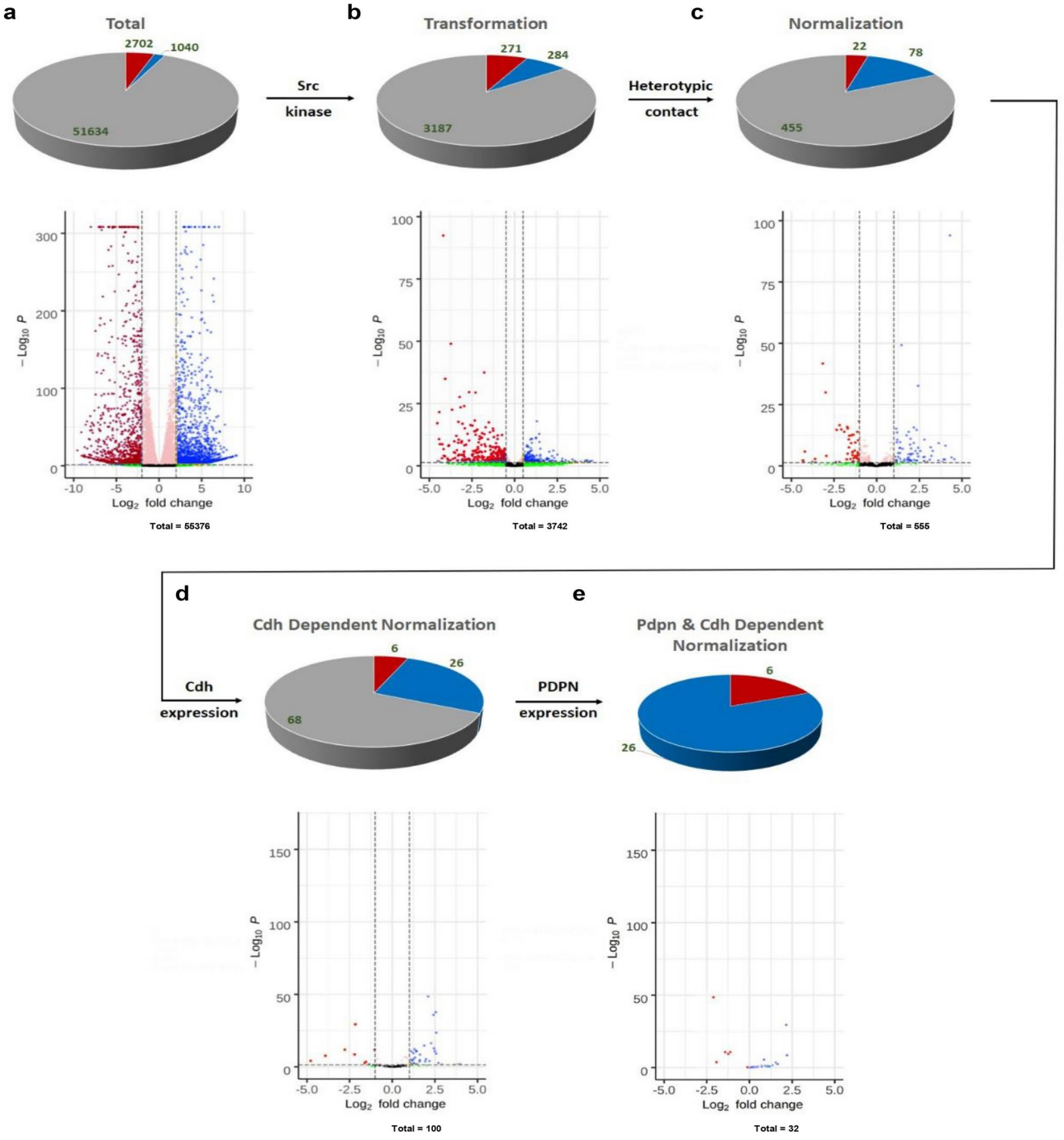


Fig. 2. Effect of contact normalization on mRNA expression in Src transformed cells. (**a**) A total of 55,376 transcripts was detected in either nontransformed (Mec) or vSrc transformed (MecSrc) mouse embryonic cells. (**a**,**b**) 3742 transcripts were differentially expressed in nontransformed (Mec) cells compared to vSrc transformed (MecSrc) cells with 2702 induced by Src (red) and 1040 suppressed by Src (blue). (**b**,**c**) Contact normalization affected the expression of 555 transcripts in vSrc transformed (MecSrc) cells, with 271 decreased (red) and 284 increased (blue) by contact with adjacent nontransformed (Mec) cells, respectively. (**c**,**d**) The expression of 100 of the transcripts affected by contact normalization relied on cadherin expression in nontransformed cells; their expression was not affected by contact with cadherin deficient (MecCdhKo) cells. The expression of 22 of these transcripts was decreased (red), while 78 were increased (blue) by contact with nontransformed cells, respectively. (**d**,**e**) Forced PDPN expression affected the expression of 32 transcripts associated with cadherin dependent contact normalization. The expression of 6 of these transcripts was increased (red) in vSrc transformed cells expressing exogenous PDPN (MecSrcPdpn) compared to vSrc transformed cells without forced PDPN expression (MecSrc), while the expression of 26 transcripts was suppressed (red) in this comparison.

PDPN transfected Src transformed (MecSrcPdpn) mouse embryonic cells cultured with themselves or in contact with other cells in the layered culture system is shown in Fig. 3.

The 26 genes in PDPN transfected vSrc transformed (MecSrcPdpn) cells that were suppressed by contact with nontansformed (Mec) cells shown in Fig. 3 were subtracted from the 78 putative suppressers involved in contact normalization of vSrc transformed (MecSrc) cells without forced PDPN expression shown in Fig. 2c. Genes in this list were then selected with expression levels of at least 3 transcripts per million in transformed (MecSrc) or nontransformed (Mec) cells. These 41 putative tumor suppressers and 6 promoters and the effects of transformation, cadherin mediated contact normalization, cadherin independent contact, and PDPN driven contact normalization on their relative expression levels are shown in Fig. 4a.

The 6 most differently expressed putative tumor promoters and suppressers regulated by cadherin dependent contact normalization and PDPN expression in Src transformed cells were selected by this filtration strategy. These suppressers include LIM homeobox 8 (Lhx8), 5-hydroxytryptamine receptor 1B (Htr1b), four and a half LIM domains 1 (Fhl1), solute carrier family 6 member 17 (Slc6a17), vestigial like family member 3 (Vgll3), and basic helix-loop-helix family member E22 (Bhlhe22). The promoters include transmembrane protein 45A (Tmem45a), aquaporin 5 (Aqp5), lymphocyte antigen 6 complex locus C1 (Ly6c1), aspartoacylase (Aspa), RIKEN cDNA 5430425K12 gene (5430425k12Rik), and podoplanin (Pdpn). Their mRNA expression levels in nontransformed (Mec), cadherin deficient (MecCdhKo), vSrc transformed (MecSrc), and PDPN transfected vSrc transformed (MecSrcPdpn) cells cultured with themselves or in contact with other cells in the layered culture system are shown as transcripts per million (TPM) in Fig. 4b. β-actin (Actb) mRNA levels were used as a control for this quantitation and were not affected by contact normalization as shown in Fig. 4b.

Contact normalization affects protein phosphorylation events in Src transformed cells

Having identified effects of cadherin mediated contact normalization on gene expression in Src transformed cells, we sought to investigate phosphorylation events modulated by this process. In particular, we sought to identify Src mediated phosphorylation events inhibited by contact normalization. Protein isolated from nontransformed, transformed, and contact normalized transformed cells was investigated by phosphoproteomic analysis to identify these events.

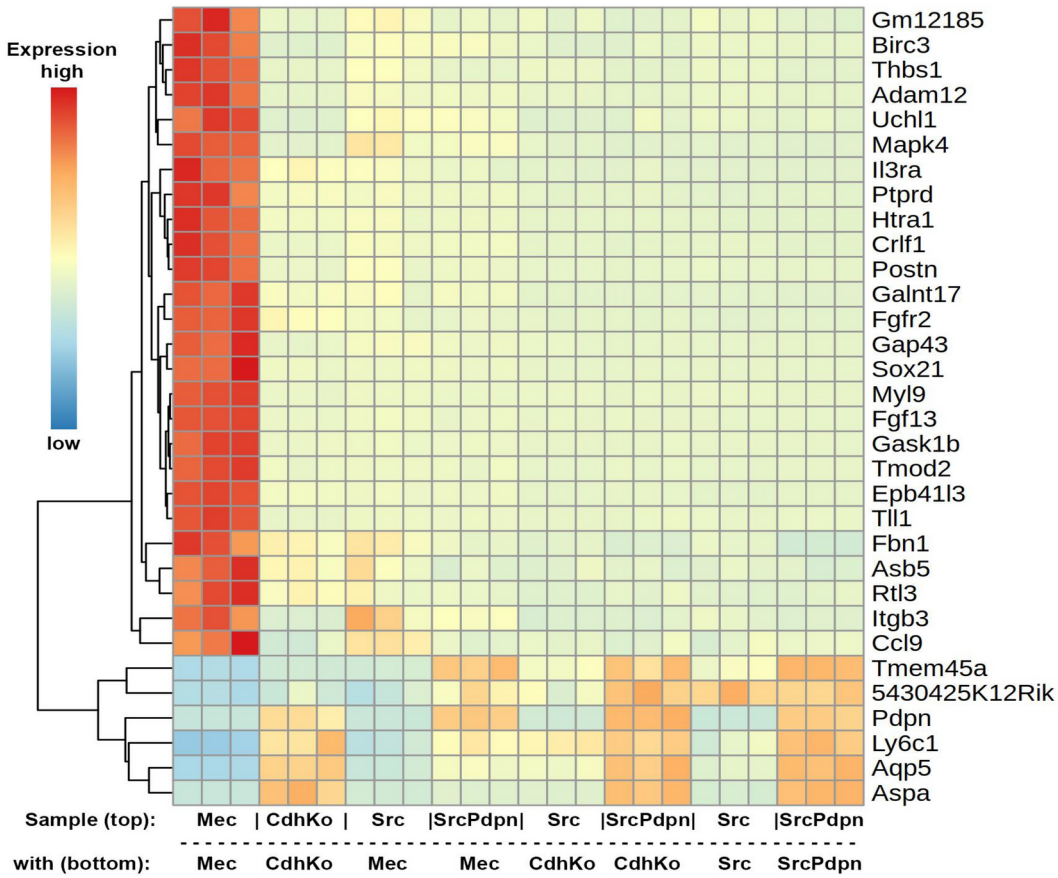


Fig. 3. Heat map and dendrogram of transcripts regulated by cadherin dependent contact normalization in presence of forced PDPN expression in Src transformed cells. Relative expression in nontransformed (Mec), cadherin deficient (MecCdhKo), vSrc transformed (MecSrc), and PDPN transfected vSrc transformed (MecSrcPdpn) mouse embryonic cells cultured with themselves or in contact with other cells in the layered culture system as indicated.

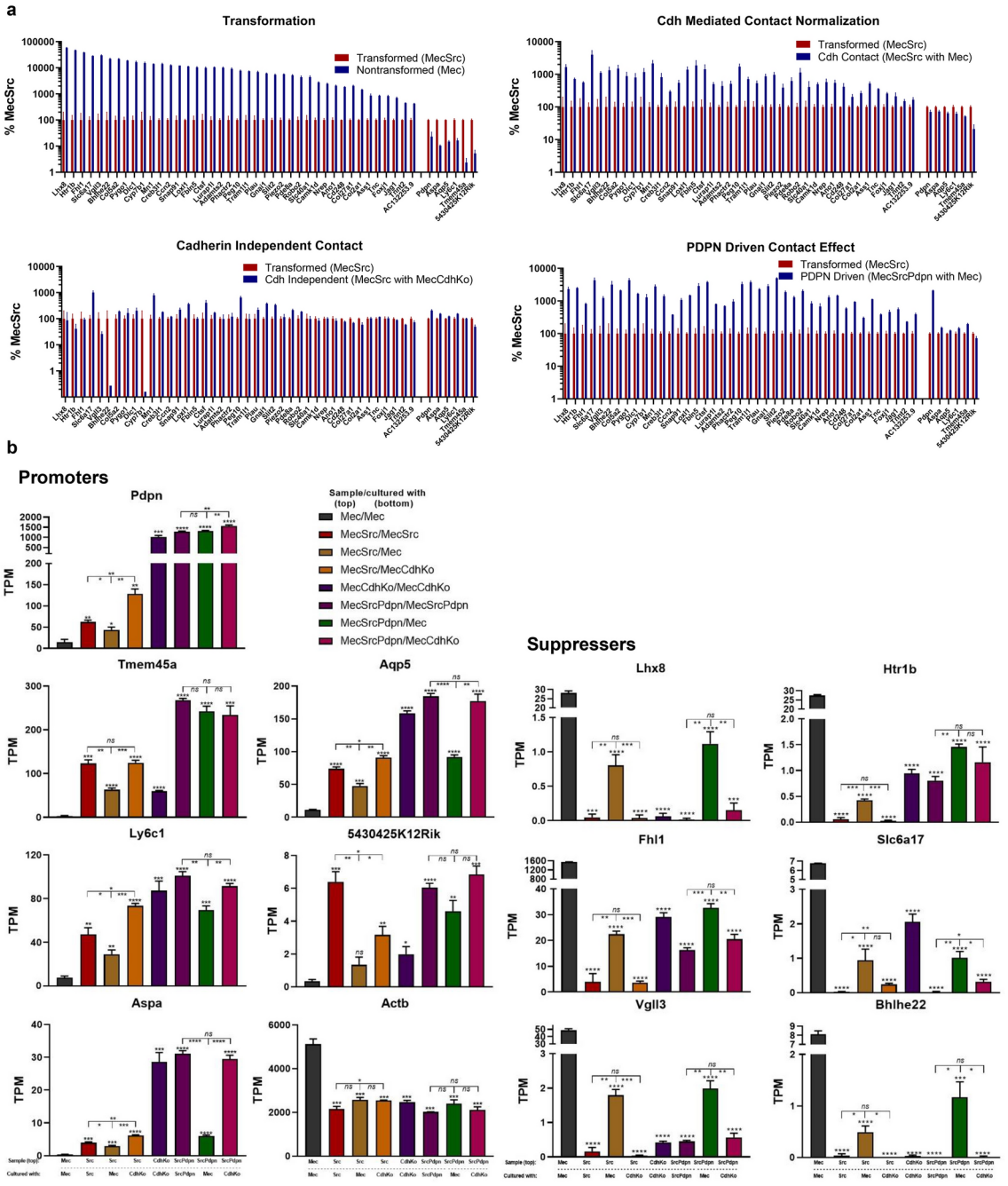
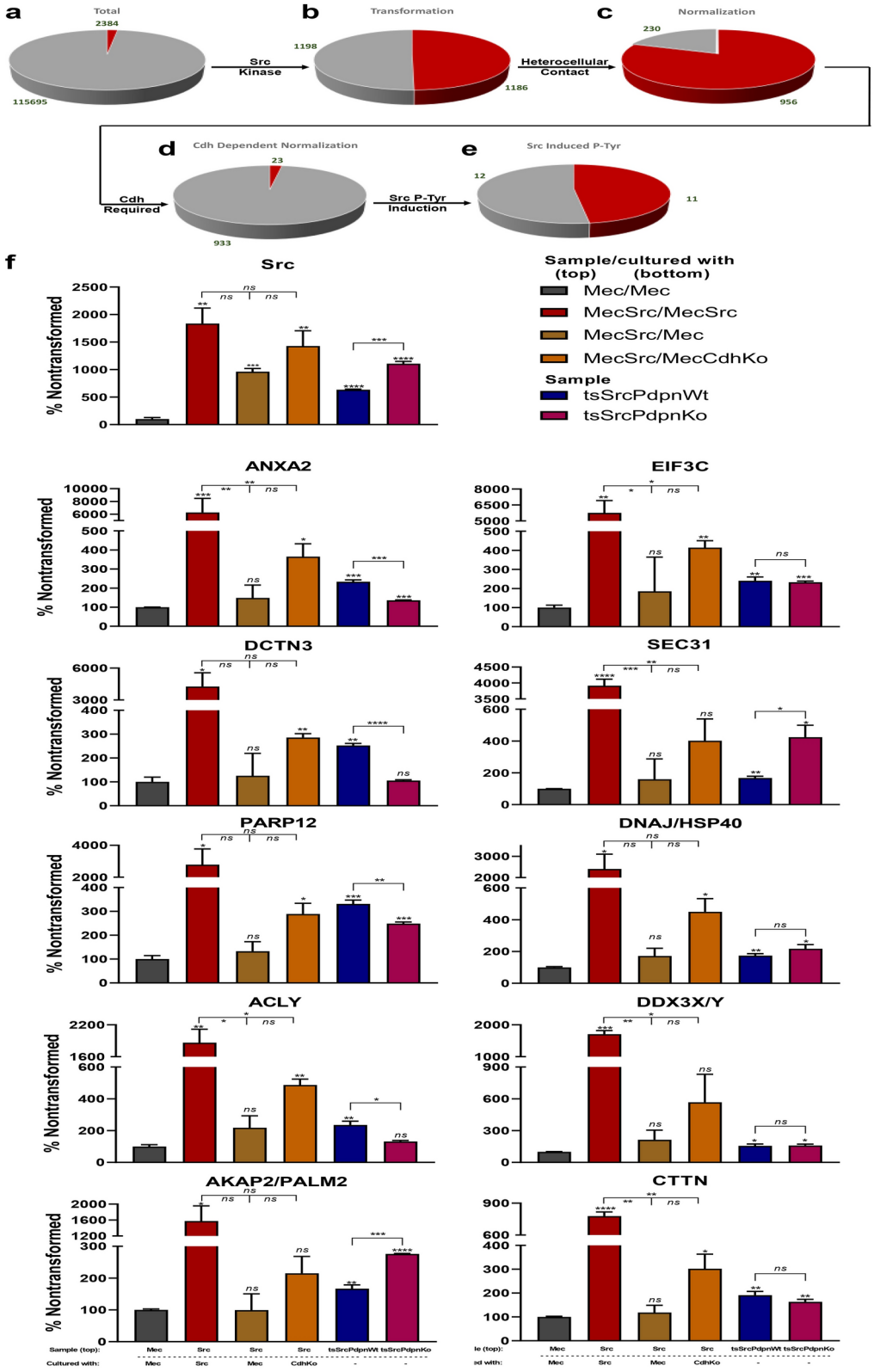


Fig. 4. Genes in Src transformed cells affected by cadherin dependent contact normalization and endogenous PDPN expression. (**a**) These genes consist of 26 putative tumor suppressers identified in Pdpn transfected vSrc transformed (MecSrcPdpn) cells shown in Fig. 2 subtracted from the 78 suppressers involved in contact normalization of vSrc transformed (MecSrc) cells without forced PDPN expression in Fig. 2c with at least 3 TPM in transformed (MecSrc) or nontransformed (Mec) cells. Effects of transformation are represented by nontranfsormed (Mec) cells, cadherin mediated contact normalization are represented by vSrc transformed (MecSrc) cells cultured with nontransformed (Mec) cells, cadherin independent contact are represented by vSrc transformed (MecSrc) cells cultured with nontransformed cadherin knockout (MecCdhKo) cells, and PDPN driven contact effects are represented by vSrc transformed Pdpn transfected (MecSrcPdpn) cells cultured with nontransformed (Mec) cells. Data are shown as percent of Src transformed (MecSrc) cells grown alone (mean + SEM, n = 3). (**b**) Expression of genes in nontransformed (Mec), cadherin deficient (MecCdhKo), vSrc transformed (MecSrc), and PDPN transfected vSrc transformed (MecSrcPdpn) cells cultured with themselves or in contact with other cells in the layered culture system are shown as transcripts per million (mean + SEM) with ns, single, double, triple, and quadruple asterisks representing $p > 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ by t-test compared to nontransformed (Mec) cells alone or between groups as indicated.



A total of 118,079 phosphorylated peptides was detected in either nontransformed (Mec) or vSrc transformed (MecSrc) cells cultured with themselves for 24 h in the layered culture system as shown in Fig. 5a. Src activity induced phosphorylation of 2384 of these peptides. Phosphorylation of these peptides was at least 500% higher in vSrc transformed (MecSrc) cells compared to nontransformed (Mec) cells cultured with themselves as shown in Fig. 5a,b.

Contact normalization decreased the phosphorylation of 1186 of the peptides phosphorylated in Src transformed (MecSrc) cells. Phosphorylation of these peptides was at least 300% lower in vSrc transformed (MecSrc) cultured with themselves compared to with nontransformed (Mec) cells as shown in Fig. 5b,c.

◀**Fig. 5**. Effect of contact normalization on protein phosphorylation in Src transformed cells. (**a**,**b**) A total of 118,079 phosphorylated peptides was detected in either nontransformed (Mec) or vSrc transformed (MecSrc) mouse embryonic cells cultured with themselves in the layered culture system for 24 h. Phosphorylation of 2384 of these peptides was increased in vSrc transformed (MecSrc) cells compared to nontransformed (Mec) cells. (**b**,**c**) Contact normalization decreased the phosphorylation of 1186 peptides in vSrc transformed (MecSrc) cells cultured with nontransformed (Mec) cells. (**c**,**d**) Phosphorylation of 956 of the peptides affected by contact normalization relied on cadherin expression in nontransformed cells (red); their phosphorylation was not affected by contact with cadherin deficient (MecCdhKo) cells. (**d**,**e**) Phosphorylation of 23 of the peptides regulated by cadherin dependent contact normalization was increased within 1 h of induction of Src kinase activity at the permissive temperature (red) in cells expressing temperature sensitive Src (tsSrcMec) compared to tsSrc transformed cells at the nonpermissive temperature. (**e**) 11 proteins containing phosphopeptides induced within 1 h by Src and regulated by cadherin dependent contact normalization contained phosphorylated tyrosines. (**f**) Phosphorylation of peptides in nontransformed (Mec), cadherin deficient (MecCdhKo), and vSrc transformed (MecSrc) cells cultured with themselves or in contact with other cells in the layered culture system for 24 h, as well as tsSrc transformed cells with (tsSrcPdpnWt) and without (tsSrcPdpnKo) forced PDPN expression for 1 h at the permissive temperature was examined. Data are shown as the percent of phosphorylation (mean + SEM, $n \geq 2$) in nontransformed (Mec) cells cultured with themselves, and vSrc transformed (MecSrc) cells cultured with themselves, cadherin competent nontransformed (Mec) cells, or cadherin deficient nontransformed (MecCdhKo) cells compared to nontransformed (Mec) cells, while tsSrc transformed cells are shown as percent of phosphorylation 1 h after shift to the permissive (34 °C) temperature compared to nonpermissive (39 °C) temperature, with ns, single, double, triple, and quadruple asterisks representing $p > 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ by t-test compared to nontransformed cells or between groups as indicated.

Phosphorylation of 956 of the peptides affected by contact normalization relied on cadherin expression in nontransformed cells. Their phosphorylation was at least 200% lower in vSrc transformed (MecSrc) cells cultured with cadherin competent nontransformed (Mec) cells compared to cadherin deficient nontransformed (MecCdhKo) cells as shown in Fig. 5c,d.

Protein from temperature sensitive Src (tsSrc) transformed PDPN transfected (tsSrcPdpnWt) cells was analyzed to find potential Src phosphorylation targets affected by contact normalization. Phosphorylation of 23 of the peptides regulated by cadherin dependent contact normalization was at least 50% greater within 1 h of induction of Src kinase activity at the permissive temperature in cells expressing temperature sensitive Src (tsSrcPdpnWt) compared to the nonpermissive temperature as shown in Fig. 5d,e. Phosphorylated tyrosine residues were found on 11 of these peptides as shown in Fig. 5e.

The 11 peptides that are initially phosphorylated in Src transformed cells in a cadherin mediated contact normalization dependent manner are found in the 10 proteins shown in Table 1. Quantitated phosphorylation of these peptides in nontransformed (Mec), cadherin deficient (MecCdhKo), and vSrc transformed (MecSrc) cells cultured with themselves or in contact with other cells in the layered culture system for 24 h, as well as tsSrc transformed cells with (tsSrcPdpnWt) and without (tsSrcPdpnKo) forced PDPN expression for 1 h at the permissive temperature is shown in Fig. 5f.

The filtration strategy employed here selected 10 proteins that were phosphorylated on tyrosine in Src transformed cells. This phosphorylation event was reduced by contact normalization of vSrc transformed (MecSrc) cells cultured with nontransformed (Mec) cells to achieve levels similar to nontransformed (Mec) cells cultured with themselves. This effect was cadherin dependent since phosphorylation events were significantly higher in vSrc transformed cells cultured with cadherin deficient nontransformed (MecCdh) cells than when cultured with cadherin competent nontransformed (Mec) cells. However, only 4 of these proteins, annexin A2 (ANXA2), dynactin subunit 3 (DCTN3), Poly ADP-ribose polymerase 12 (PARP12), and ATP-citrate synthase

Symbol	Description	Sequence	Src fold change
ANXA2	Annexin A2	SYSPY*DMLESIK	63
EIF3C	Eukaryotic translation initiation factor 3 subunit C	QGTY*GGYFR	58
DCTN3	Dynactin subunit 3	YLDPEY*IDR	43
SEC31	Transport protein	AQGKPVSGQESSQSPY*ER	39
PARP12	Poly ADP-ribose polymerase 12	NLVY*GTIR	28
DnaJ, HSP40	DnaJ HSP chaparone	AIYDIY*GK(R)	24
ACLY	ATP-citrate synthase	TTDGVY*EGVAIGGDR	19
DDX3X/Y	ATP-dependent RNA helicase	GDY*DGIGGR	17
AKAP2, PALM2	A-kinase anchor protein, Paralemmin	EGPY*SEPSK	16
CTTN	Cortactin	ASHGY*GGK	8

Table 1. Src kinase mediated phosphorylation events inhibited by cadherin dependent contact normalization. Protein symbol, name, and tyrosine phosphorylation sites are shown along with fold change in Src transformed (SrcMec) cells cultured with themselves compared to nontransformed (Mec) cells cultured with themselves.

(ACLY), were phosphorylated in a PDPN dependent manner. These proteins were phosphorylated more in PDPN expressing tsSRC transformed (tsSrcPdpnWt) cells then PDPN deficient tsSrc transformed (tsSrcPdpnKo) cells one hour after shift from the nonpermissive (39 °C) to the permissive (34 °C) temperature as shown in Fig. 5f.

Src kinase phosphorylation at Y^{416} (LIEDNEY*TAR) was used as a control for this phosphoproteomic quantitation strategy. Increased phosphorylation was seen at this site in vSrc transformed (MecSrc) cells as expected, and these levels were not significantly affected by contact with cadherin competent (Mec) or cadherin deficient (MecCdhKo) cells. This phosphorylation event was also induced in tsSrc transformed cells within 1 h after shift to the permissive temperature. Interestingly, this effect was higher in PDPN deficient (tsSrcPdpnKo) cells than PDPN expressing (tsSrcPdpnWt) cells as shown in Fig. 5f.

Oncogenic Src kinase activity induces Aspa, Ly6c1, and PDPN protein expression and Anxa2 phosphorylation

We utilized Western blotting to investigate the expression of gene products affected by contact normalization identified by RNA-Seq and LC–MS/MS in this study. RNA-Seq identified 6 putative tumor promoters induced by Src kinase activity and suppressed by contact normalization. These include PDPN, Tmem45a, Aqp5, Ly6c1, 5430425k12Rik, and Aspa as shown in Fig. 4b. We were not able to obtain antisera specific for 5430425k12Rik, which was expressed at very low levels of below 8 TPM in any cell type. Aqp5 and Tmem45a expression was not detected by Western blot analysis. In contrast to these proteins, Western blot results indicate that Src kinase activity induced PDPN, Ly6c1, and Aspa protein expression in Mec cells as shown in Fig. 6.

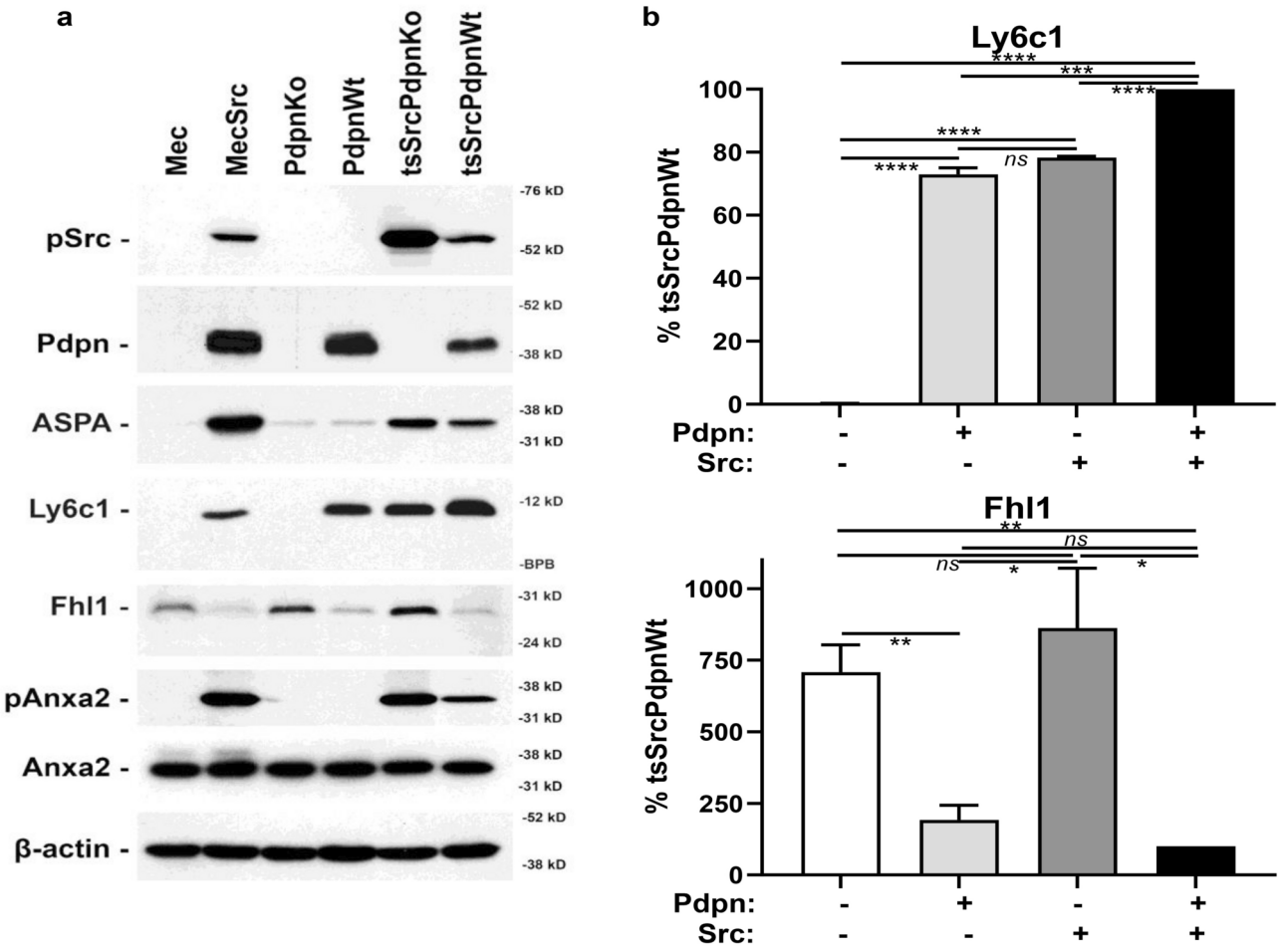


Fig. 6. Protein expression in transformed and nontransformed cells. (**a**) Active Src (phosphorylated at Tyr 416), PDPN, ASPA, Ly6c1, Fhl1, Anxa2, Anxa2 phosphorylated at Tyr 238, and β-actin were detected by Western blot analysis of protein from nontransformed (Mec) cells and vSrc transformed (MecSrc) cells, as well as nontransformed homozygous null PDPN knockout cells with (PdpnWt) or without (PdpnKo) expression, and tsSrc transformed homozygous null PDPN knockout cells with (tsSrcPdpnWt) and without (tsSrcPdpnKo) forced PDPN expression cultured for 24 h at the permissive (34 °C) temperature. (**b**) Ly6c1 and Fhl1 expression was quantitated and shown as the percent of tsSrcPdpnWt cells (mean + SEM) with ns, single, double, triple, and quadruple asterisks representing $p > 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ by t-test between groups as indicated.

LC–MS/MS identified 10 putative tumor promoters phosphorylated by Src in a manner suppressed by contact normalization. These include Anxa2, Eif3c, Dctn3, Dex31, Parp12, Dnaj/Hsp40, Acly, Ddx3x/y, Akap2/Palm2, and Cttn as shown in Table 1 and Fig. 5f. Western blotting found robust and comparable expression of all of these proteins in nontransformed (Mec) and vSrc transformed (MecSrc) cells (data not shown). We were able to obtain phosphospecific antisera for Anxa2, but not the other proteins. Anxa2 phosphorylation at Tyr 238 was identified as the most profound Src mediated phosphorylation event by phosphoproteomic analysis, at over 60 fold higher in vSrc transformed (MecSrc) cells than nontransformed (Mec) cells as shown in Table 1 and Fig. 5f. This event was confirmed by Western blot analysis with phosphospecific antisera as shown in Fig. 6a. These data are consistent with reports that Src signaling inducing Anxa2 phosphorylation at Tyr 238 [44,45].

Contact normalization suppresses PDPN expression in transformed cells, and PDPN expression can override this effect and enable cells to escape from this form of growth control [4,21]. Therefore, we sought to investigate the effects of PDPN signaling on the expression of the proteins affected by contact normalization that were identified in this study. LC–MS/MS findings shown in Fig. 5 were filtered from data obtained in cells with tsSrc activated for 1 h to find most direct Src substrates. Here, we used Western blotting to examine protein expression at 24 h after tsSrc kinase activation in cells with and without PDPN to identify functionally relevant Src effector targets. These included nontransformed PDPN knockout cells with (PdpnWt) and without (PdpnKo) forced PDPN expression, and tsSrc transformed cells with (tsSrcPdpnWt) and without (tsSrcPdpnWt) forced PDPN expression. Western blotting confirmed appropriate Src kinase activity and PDPN expression in these as shown in Fig. 6.

While Src kinase activity increased Aspa expression, and Anxa2 phosphorylation at Tyr 238, these events were not PDPN dependent. PDPN expression did not affect Aspa expression or Anxa2 phosphorylation in nontransformed (PdpnKo or PdpnWt) cells or tsSrc transformed (tsSrcPdpnKo or tsSrcPdpnWt) cells as shown in Fig. 6. However, in contrast to Aspa expression and Anxa2 phosphorylation, both PDPN and Src kinase activity did increase Ly6c1 expression. Ly6c1 expression was $1.2\times10^6\pm7.3\times10^5$ fold higher in nontransformed (PdpnWt) cells with PDPN expression, $1.3\times10^6\pm7.9\times10^5$ fold higher tsSrc transformed (tsSrcPdpnKo) cells without PDPN expression, and $1.7\times10^6\pm1.0\times10^6$ (mean + SEM with n = 3) fold higher in tsSrc transformed (tsSrcPdpnWt) cells with PDPN expression than nontransformed (PdpnKo) cells without PDPN expression as shown in Fig. 6. Ly6c1 has been identified as a tumor promoter associated with cancer progression along with PDPN in tumor cells and cancer associated fibroblasts (CAFs) [46,47] (see "Discussion").

Fhl1 has been identified as a tumor suppresser with expression that is inhibited by Src kinase activity and induced by contact normalization [36,48]. Results from this study confirm this expression pattern. Fhl1 expression was 28.4 ± 7.8 fold lower in nontransformed (PdpnWt) cells with PDPN expression, 118 ± 17 fold lower tsSrc transformed (tsSrcPdpnKo) cells without PDPN expression, and 14.7 ± 2.2 (mean + SEM with n = 3) fold lower in tsSrc transformed (tsSrcPdpnWt) cells with PDPN expression than nontransformed (PdpnKo) cells without PDPN expression as shown in Fig. 6.

Contact normalization inhibits Src kinase induced Wnt signaling

We utilized a functional (TopGfp) and nonfunctional (FopGfp) β-catenin driven GFP reporter construct to analyze Wnt signaling in nontransformed (FopGfpMec and TopGfpMec) and vSrc transformed (FopGfpMecSrc and TopGfpMecSrc) cells. Src transformed cells expressing the functional construct (TopGfpMecSrc) displayed GFP expression indicative of Wnt signaling as shown in Fig. 7a,b. In contrast, this GFP expression was not seen in nontransformed cells expressing the nonfunctional (FopGfpMec) or functional (TopGfpMec) construct as shown in Fig. 7a,b. Furthermore, this β-catenin driven GFP expression was increased by the Wnt agonist LiCl [49] and decreased by the Wnt antagonist IWP-2 [50]. LiCl increased GFP intensity in transformed cells (TopGfpMecSrc) by over 65% ($p<0.05$ by t-test with n = 5), while IWP-2 decreased GFP intensity by over 80% ($p<0.0001$ by t-test with n = 5), as shown in Fig. 7a,c. LiCl also induced PDPN expression in Src transformed (MecSrc) cells by over 300% as shown in Fig. 7f. These data are consistent with Wnt signaling inducing PDPN expression in Src transformed cells.

Having demonstrated Wnt signaling in Src transformed (TopGfpMecSrc) cells, we sought to explore the effects of contact normalization on β-catenin driven GFP expression. β-catenin driven GFP expression in Src transformed (TopGfpMecSrc) cells was decreased by over 80% ($p<0.01$ with n = 3 by t-test) when cultured with cadherin competent (Mec) cells compared to transformed cells cultured with themselves. In contrast, this GFP expression was not significantly changed when cultured with cadherin deficient (MecCdhKo) nontransformed cells as shown in Fig. 7d,e. These data indicate that cadherin mediated contact normalization inhibited β-catenin driven GFP expression in Src transformed cells ($p<0.01$ by t-test with n = 3) as shown in Fig. 7e.

Discussion

The Src tyrosine kinase phosphorylates effectors to promote tumor cell growth and motility [3–5]. Nontransformed cells form heterocellular cadherin junctions with Src transformed cells to normalize tumor cell growth and morphology by contact normalization [22–24,51]. Nontransformed cells utilize this process to completely inhibit the growth of adjacent Src transformed cells used in this study [21,25]. The PDPN receptor enables transformed cells to escape from this form of growth control, called "contact normalization", in order to become invasive and metastatic [4,11,18,21,22,35]. Here, we present mRNA and phosphoproteomic analyses to identify genes and proteins involved in this process.

Oncogenic Src kinase activity altered the expression of ~ 7% genes identified by RNA-Seq in this study by at least fourfold. Src induced the expression of ~ 72% of these transcripts. Contact normalization inhibited the expression of ~ 7% of these Src induced genes, which are considered potential tumor promoters in this study. Conversely, contact normalization increased the expression of ~ 8% of the genes suppressed by Src, which are

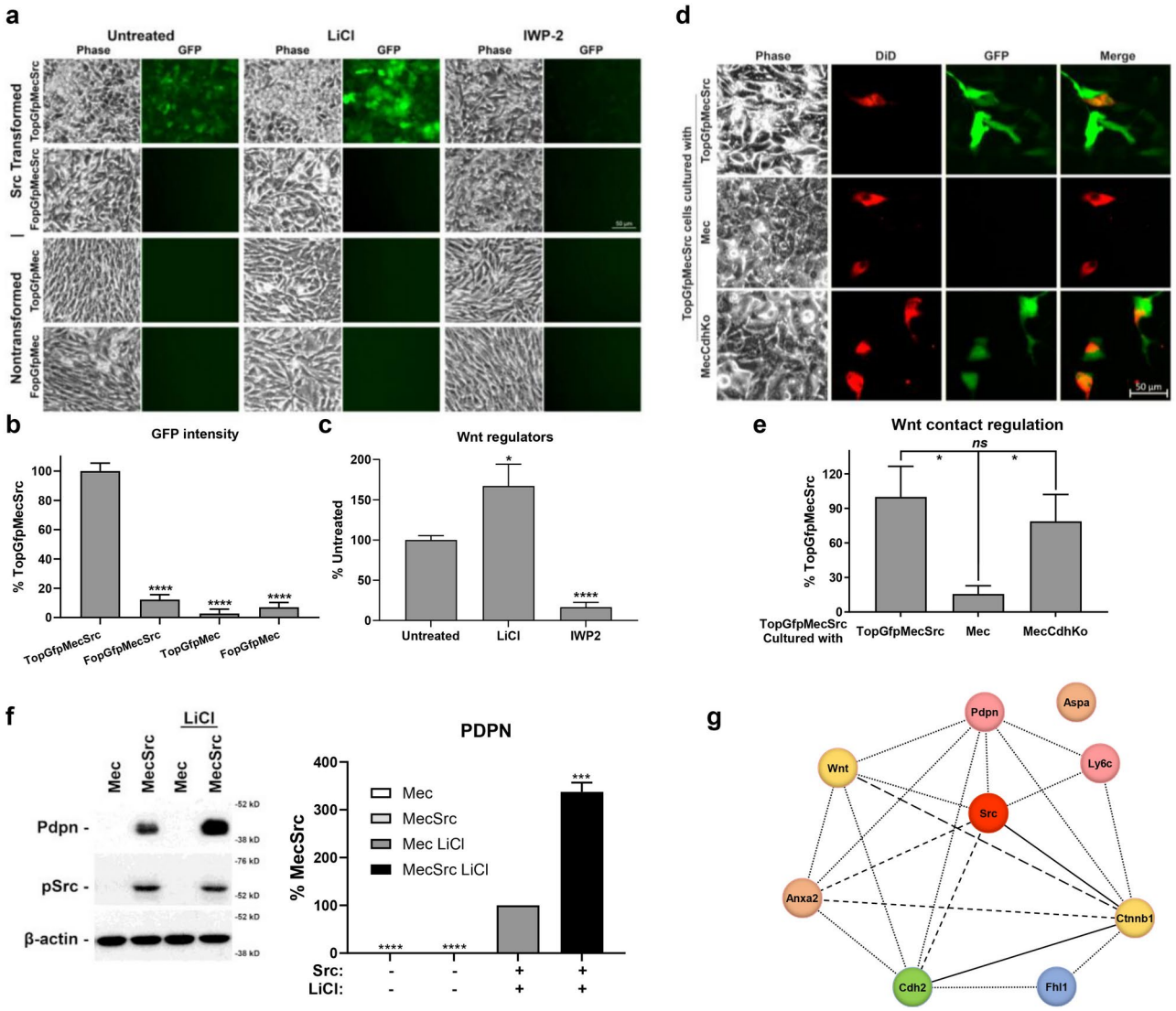


Fig. 7. Effect of contact normalization on Src kinase mediated Wnt signaling and PDPN expression. (**a**) Nontransformed (TopGfpMec) and Src transformed (TopGfpMecSrc) cells expressing a functional β-catenin driven GFP reporter construct or nonresponsive construct (FopGfpMec and FopGfpMecSrc) were cultured 48 h with or without 10 mM LiCl or 10 uM IWP-2, and observed by phase contrast and fluorescence microscopy as indicated. (**b**) Mean GFP intensity of representative untreated cells was normalized to the percent GFP signal in Src transformed cells expressing the responsive β-catenin driven GFP reporter construct (TopGfpMecSrc). Data are shown as mean + SEM (n = 5). (**c**) Mean GFP intensity of representative cells was normalized to the percent GFP signal in untreated Src transformed (TopGfpMecSrc) cells. Data are shown as mean + SEM (n = 5). (**d**) TopGfpMecSrc cells were labeled with DiD (red) and cultured with nonlabelled Src transformed (TopGfpMecSrc) cells, cadherin expressing nontransformed (Mec) cells, or cadherin deficient nontransformed (MecCdhKo) cells with 10 mM LiCl for 48 h, and visualized by phase contrast and fluorescence microscopy as indicated. (**e**) Mean GFP intensity of representative cells was normalized to the percent GFP signal in Src transformed (TopGfpMecSrc) cells cultured with themselves. Data are shown as mean + SEM (n = 3). Scale bar = 50 microns for all panels, and single, double, triple, and quadruple asterisks represent $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ by t-test, respectively. (**f**) Active Src (phosphorylated at Tyr 416), PDPN, and β-actin were detected by Western blot analysis of protein from nontransformed (Mec) cells and vSrc transformed (MecSrc) cells cultured with 10 mM LiCl for 10 h. PDPN expression was quantitated and shown as the percent of MecSrc cells (mean + SEM) with triple and quadruple asterisks representing $p < 0.001$ and $p < 0.0001$ compared to nontreated MecSrc cells by t-test as indicated. (**g**) Proteins most affected by contact normalization. Oncogenic Src kinase activity disrupts Cdh2 junctions and inhibits Fhl1 expression. Src kinase activity also activates β-catenin mediated Wnt signaling and increases Anxa2, Pdpn, Aspa, and Ly6c1 expression. Cadherin dependent contact normalization most notably inhibits Pdpn and Ly6c1 expression and induces Fhl1 expression in Src transformed cells. Protein interaction networks were identified using STRING (https://string-db.org/) with a confidence score of 0.150 (Low), 0.400 (Medium), 0.700 (High) and 0.900 (Highest) indicated by dotted, short dashed, long dashed, and solid lines, respectively.

considered tumor suppressers in this study. These data indicate that cadherin mediated contact normalization alters the expression of ~ 1% of the transcripts expressed by Src transformed cells.

These results are consistent with previous studies performed with nucleotide microarray technology. Seminal reports found that oncogenic Src kinase activity increased the expression of ~ 6% and decreased the expression of ~ 2% of the genes in the transcriptome. Contact normalization increased the expression of ~ 2.3% and decreased the expression of ~ 0.2% of the Src modulated genes in these reports, which identified Fhl1 as a functional tumor suppresser induced by contact normalization [25,36,43]. A subsequent report utilized nucleotide microarrays to find that Src increased and decreased the expression of ~ 12% of genes in the transcriptome, and contact normalization increased or decreased the expression of ~ 0.2% of these Src modulated genes. This report also identified Fhl1 as a functional tumor suppresser induced by contact normalization. In addition, this report found Src induces PDPN expression to promote cell motility [35]. More recent data obtained by RNA-Seq find that Src kinase activity increased expression of ~ 10% and decreased expression of ~ 5% of genes in the transcriptome. Contact normalization affected the expression of ~ 20% of these genes affected by Src activity, with regulation of ~ 26% of these genes dependent on the formation of heterocellular cadherin junctions. Thus, cadherin mediated contact normalization altered the expression of ~ 0.4% of the transcripts expressed by Src transformed cells. PDPN was confirmed as a tumor promoter, and Fhl1 was confirmed as a tumor suppresser effected by contact normalization in that study [21].

The present study is the first we are aware of to combine nonbiased phosphoproteomics and RNA-Seq to study genes affected by contact normalization. Src induced phosphorylation of 2384 or ~ 2% of the 118,079 phosphorylated peptides found in this study. Cadherin dependent contact normalization inhibited 956 or ~ 40% of these Src induced phosphorylation events. A temperature inducible construct was used in this study to find that only 23 or ~ 2.4% of these peptides were phosphorylated within 1 h of Src kinase activation. Ten of these proteins were phosphorylated on tyrosine, and only 4 of these events were augmented by PDPN expression. These proteins, namely Anxa2, Cdtn3, Parp12, and Acly represent the most prominent and initial Src induced phosphorylation targets augmented by PDPN and suppressed by contact normalization. These data exceed previous reports that identified 13,628 phosphopeptides in Src transformed cells, but which also found Parp12 phosphorylation to be an early event in the PDPN assisted Src transformation process [30].

Antisera were available to examine Anxa2 phosphorylation, but not the other proteins to verify protein phosphorylation events in cells by Western blotting in this study. Annexins are calcium dependent phospholipid binding proteins. ANXA2 is a dynamic family member that interacts with a variety of other proteins including the PDPN binding partner CD44 to control cell growth and motility. ANXA2 expression is associated with many cancers [52,53]. In addition to tissue expression, ANXA2 is found in circulating tumor cells and as a soluble biomarker in cancer patient serum that inversely correlates with patient survival [53,54]. Although not PDPN dependent, Src clearly induced Anxa2 phosphorylation on Tyrosine 238. These data are consistent with previous reports that Src signaling induces Anxa2 phosphorylation at Tyr 238 [44,45]. Indeed, Src phosphorylates Anxa2 to promote tumor cell growth and motility leading to cancer progression. For example, Src phosphorylates ANXA2 on Tyr23 to drive it to the plasma membrane where it interacts with actin to promote cell motility [55–57]. ANXA2 has been shown to induce Wnt/β-catenin signaling to promote tumor cell growth and resist chemotherapy and radiation treatments [58–60].

Taken together, data from this study indicate that Src induces Wnt signaling and PDPN expression, and phosphorylates proteins including Anxa2 and others listed in Table 1 in order to regulate expression of genes that enable transformed cells to override cadherin mediated contact normalization. In particular, this study identified PDPN and Ly6c as tumor promoters and Fhl1 as a tumor suppresser in this process. The roles of PDPN as a tumor promoter and Fhl1 as a tumor suppresser that act downstream of Src signaling in contact normalization have been previously described [4,18,36,48]. This is the first report we know of to implicate Ly6c1 in contact normalization. However, Ly6c1 has been identified as a tumor promoter associated with cancer progression along with PDPN in tumor cells and cancer associated fibroblasts (CAFs) [46,47].

Conclusion

This study is the first we know of to find that contact normalization suppresses β-catenin mediated Wnt signaling downstream of oncogenic Src kinase activity. In addition, LiCl induced Wnt signaling and PDPN expression in Src transformed cells, but not nontransformed cells. These data indicate that Src utilizes Wnt signaling to induce PDPN expression, but that Wnt signaling is not sufficient to induce PDPN expression in the absence of oncogenic Src kinase activity. Previous findings implicate crosstalk between Src, Wnt, and PDPN in oncogenic signaling events. For example, Src phosphorylates LRP5 to regulate Wnt signaling, and Wnt induces GSK3β phosphorylation to increase Src activity leading to cancer progression [61]. Wnt signaling also induces PDPN expression in human lung epithelial cells [62], and PDPN potentiates β-catenin mediated Wnt signaling to drive mammary tumorigenesis [63]. Agents that target PDPN have been shown to inhibit the motility and tumor progression of a variety of cancer cells including melanoma [64,65], glioma [66], and squamous cell carcinoma [4,18,67,68]. Results from this study implicate β-catenin mediated Wnt signaling, PDPN, Ly6c1, and Fhl1 in contact normalization of Src transformed cells as shown in Fig. 7g. Further investigations of these interactions are bound to elucidate mechanisms that drive cancer progression, biomarkers that can be used to better diagnose malignancies, and methods that can be used to combat tumorigenesis and cancer progression.

Data availability

Research material and data described in this study are available from the corresponding author and MassIVE data set number MSV00009421 at ftp://massive.ucsd.edu/v07/MSV000094121, and the Sequence Read Archive

(SRA) database under the accession number PRJNA1070015 at https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1070015.

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Author contributions

R.E.N., A.A.G., and C.M.L. generated cells with β-catenin reporter constructs and analyzed their gene and protein expression. C.H. and A.C.Y. analyzed protein expression in nontransformed and transformed cells. E.P.R. generated PDPN competent and deficient cells expressing temperature sensitive Src kinase constructs and analyzed their gene and protein expression. S.A.S. generated nontransformed and Src transformed cadherin competent and deficient cells and analyzed their gene and protein expression. J.T. analyzed gene expression obtained by RNA-Seq. C.Z. and H.Z. analyzed protein and data obtained by LC–MS/MS. G.S.G. assisted with cell culture, data analysis, protein and gene expression experiments, and directed all aspects of the study and manuscript preparation. All authors assisted with data analysis, manuscript preparation, and figures.

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Competing interests

GSG has intellectual property and ownership in Sentrimed, Inc., and REN, CH, and ACY received financial support from Sentrimed, Inc. which is developing agents that target Pdpn to treat diseases including cancer and arthritis. The remaining authors declare no conflicts of interest.

Additional information

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