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RESEARCH ARTICLE

Migration inhibition of mammary epithelial cells by Syk is blocked in the presence of DDR1 receptors

Brit Neuhaus · Sebastian Bühren · Barbara Böck · Frauke Alves · Wolfgang F. Vogel · Friedemann Kiefer

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Abstract The non-receptor tyrosine kinase Syk is a well-characterized hematopoietic signal transducer, which is also expressed in non-hematopoietic cells. In epithelial cells, the function of Syk is not wholly known. It interacts with the receptor tyrosine kinase DDR1 and is frequently lost from metastatic mammary tumors. Here, using genetic tracing, we demonstrate Syk expression in murine mammary epithelium, myoepithelium and skin epithelium, but not in intestinal or lung epithelia. Investigating possible functions of Syk, we found a substantial suppression of cell mobility that depended on Syk kinase activity in trans-well migration and wounding assays. Co-expression of DDR1 resulted in constitutive interaction and strong activation of Syk kinase. Most importantly, Syk-mediated migration inhibition was blocked in the presence of DDR1, while conversely DDR1 knockdown restored migration inhibition. Our study identifies Syk as a potent inhibitor of epithelial migration and describes a first functional consequence of the interaction with the collagen receptor DDR1.

Keywords Syk · DDR1 · Migration · Epithelial cells · Cancer

Introduction

All inner and outer body surfaces are formed by static, polarized sheets of epithelial cells, which are non-motile and form tight cell-cell and cell-matrix contacts. Tumors

B. Neuhaus and S. Bühren contributed equally to this work.

Wolfgang F. Vogel (deceased on 5 December 2007).

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B. Neuhaus · S. Bühren · B. Böck · F. Kiefer (✉)
Department of Vascular Cell Biology,
Max Planck Institute for Molecular Biomedicine,
Röntgenstr. 20, 48149, Münster, Germany
e-mail: fkiefer@gwdg.de

F. Alves
Department of Hematology and Oncology,
University Medical Center Göttingen, Göttingen, Germany

F. Alves
Department of Molecular Biology of Neuronal Signals,
Max Planck Institute of Experimental Medicine,
37075 Göttingen, Germany

W. F. Vogel
Department of Laboratory Medicine and Pathobiology,
University of Toronto, Medical Science Building, Room 7334A,
1 King's College Circle, Toronto, ON M5S 1A8, Canada

Present Address:
B. Böck
Division of Vascular Oncology and Metastasis (A190),
German Cancer Research Center (DKFZ), Im Neuenheimer Feld
280, 69120 Heidelberg, Germany

Present Address:
B. Neuhaus
Department of Biological Mechanisms of Ageing, Max Planck
Institute for Biology of Ageing, 50931 Cologne, Germany

derived from the epithelia of the breast, lung, prostate and bladder constitute the majority of human cancers with high-grade malignancy and pronounced metastatic potential. Malignant transformation and ensuing metastatic capability are accompanied by profound changes in gene expression, which include oncogene activation and loss of tumor suppressors. In addition, the extracellular matrix (ECM) has been recognized to contribute to genetic changes during cancer progression [1].

The 72-kDa cytoplasmic non-receptor tyrosine kinase Syk has long been considered a hematopoietic cell-specific signal transducer. Syk is characterized by a C-terminal kinase domain, an adjacent linker region (interdomain B) and an N-terminal tandem SH2 domain. The SH2 domains are separated by an additional linker (interdomain A) and mediate leukocyte responses, such as proliferation, differentiation and phagocytosis by binding to tandem tyrosine motifs in the cytoplasmic signaling chains of classical immunoreceptors [2]. In addition, Syk has been described to be express in non-hematopoietic tissues as diverse as hepatocytes, fibroblasts, epithelial or neuronal cells [3]. However, the various roles of Syk in non-hematopoietic cell types are still poorly understood. The finding that Syk is frequently lost during mammary carcinoma progression provides evidence for an anti-oncogenic activity in non-hematopoietic cells (for review see [4]) and is very different from the pro-tumorigenic action reported for hematopoietic cells [5]. Loss of Syk, which can be mediated by promotor hypermethylation [6, 7], has been reported for a number of different tumor types and is associated with a poor prognosis in human breast cancer [4, 8, 9]. The basis for tumor suppression by Syk remains unclear. In experimental studies, Syk not only affected cell division and proliferation, but also cell invasiveness and metastatic capacity, suggesting that Syk affects very different aspects of cell behavior, including cell migration [10–13].

Discoidin domain receptor tyrosine kinases (DDRs) constitute a distinct class of collagen receptors [14], characterized by a unique extracellular domain homologous to the *Dictyostelium discoideum* lectin discoidin. Two different DDRs, DDR1 and DDR2, have been identified, with DDR1 being widely expressed in epithelial cells of the lung, colon, pancreas and mammary gland [15–17]. DDR1 is expressed in five isoforms, isoforms DDR1a to DDR1e, that are generated by alternative splicing [16, 18, 19], with the isoforms DDR1a and DDR1b being most abundant in adult tissues. The kinase-deficient DDR1d and DDR1e isoforms are differentially expressed in human colon cancer cell lines, whereas the last identified isoform DDR1c, which lacks the discoidin domain as well as parts of the adjacent stalk region, is selectively expressed in postmeiotic germ cells [19].

There is only relatively little information about DDR1 downstream signaling pathways. In addition to an inhibiting function in both cell proliferation and apoptosis [20, 21], effects on cell migration have been reported. However, depending on the cell type under investigation, inhibitory as well as migration-promoting effects have been described [22–24], suggesting that the function of DDR1 is strongly influenced by the cellular environment. Interestingly, enhanced DDR1 expression is found in various human tumors, especially fast-growing, highly invasive tumors arising from mammary, ovary and lung epithelium as well as metastatic brain tumors [17, 25].

Syk and DDR1 have been reported to interact in epithelial cells; however, the functional relevance of this interaction remains unclear [8]. We tested a potential function of Syk in the maintenance of the non-motile epithelial phenotype and correlated the action of Syk with the co-expression and hence complex formation with DDR1.

To investigate Syk expression in different mouse epithelia, we used a genetic tracing approach that allows the visualization of Syk expression with cellular resolution and high sensitivity. We observed a highly differential expression of Syk in murine epithelia. Functionally, Syk caused a profound migration inhibitory effect in transformed and non-transformed epithelial cells during chemotaxis-induced as well as spontaneous migration. In addition, we revealed a potential function for the reported interaction between Syk and DDR1, which results in strong activation of Syk kinase and a release of the Syk-mediated migration inhibition.

Our study identifies Syk as a potent migration inhibitor and defines a functional relation between Syk and DDR1 in mammary epithelial cells.

Materials and methods

Reagents

Anti-Syk antibodies N19 and 4D10, anti-DDR1 antibody C20 and anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibodies 4G10 and pY20 were from Upstate (Lake Placid, NY) and BD Biosciences (San Diego, CA), respectively. Anti-phospho-Syk (525, 526) antibody was supplied by Cell Signaling Technologies (Beverly, MA). Protein-A-HRP was obtained from Pierce (Rockford, IL), and anti-rabbit [^{35}S]-IgG was from GE Healthcare (Freiburg, Germany).

Generation of Syk-Cre-recombinase knock-in mice for lineage tracing

To visualize the expression of Syk in vivo, we expressed the Cre-recombinase under the control of the endogenous

Syk promotor. The resulting Syk-Cre *knock-in* mouse was generated by standard ES cell homologous recombination techniques [30]. Due to the embryonic lethality of homozygous Syk deletion, only mice heterozygous for the knock-in allele were available for further breeding. These mice were intercrossed with the *R26R*-YFP reporter strain, which carries the YFP gene under the control of the Rosa26 locus [31]. YFP expression in this strain is blocked by a floxed PGK-neo cassette, which is however permanently deleted in all cells expressing the Cre-recombinase, permanently marking all cells and their progeny in which the Syk promotor was ever active.

Tissues, tissue culture and cell lines

293T, Cos1, NIH 3T3, T47D, MDA-MB-468 and HB2 (kindly provided by Joyce Taylor-Papadimitriou) cells were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, UK) supplemented with 10% FCS (PAN Biotech GmbH, Germany), 100 µg/ml penicillin/streptomycin, 20 mM L-glutamine, 10 mM sodium pyruvate (PAA, Austria) and 0.001% MTG (Sigma-Aldrich). Culture medium for T47D and HB2 cells was additionally supplemented with 8 µg/ml insulin or 10 mg/ml insulin plus 5 µg/ml hydrocortisone, respectively. HC11 cells were cultured in RPMI-1640 medium (Gibco BRL, UK)/10% NCS, supplemented as described. For some experiments, cells were serum-starved in medium supplemented with 0.5% FCS or 0.5% NCS.

Transfection and retroviral transduction

Cos1 cells and 293T cells were transfected with GeneJammer® Transfection Reagent (Stratagene, USA) using 5–20 µg of total DNA. Assays were performed 36–48 h after transfection.

HC11 cells were infected with supernatants of the ecotropic packaging cell line GP + E-86 stably transfected with different pMSCV-hSyk species. Infections were repeated three times over a period of 6 days, followed by a 7-day selection with 1 µg/ml puromycin. Syk expression was confirmed by Western blotting of total cell lysates (TCL).

siRNA knockdown

All cells were transfected with a total of 200 pmol RNAi (Validated Stealth DuoPak specific for DDR1, Invitrogen). T47D cells and HB2 cells were transfected with Lipofectamine 2000 following a reverse transfection protocol. Briefly, 200 pmol RNiA and Lipofectamine were each diluted in 250 µl serum-free Opti-MEM, incubated at RT for 5 min, combined with each other and incubated for another 20 min at RT. Then 400,000 freshly harvested cells were diluted in 2 ml complete growth medium without antibiotics, seeded on a six-well plate and immediately mixed with the RNAi-Lipofectamine reagent.

MDA-MB-468 cells were transfected with Lipofectamine RNAiMax according to the manufacturer's reverse transfection protocol. Transfected cells were incubated at 37°C in a CO_2 incubator for 24–72 h. Migration assays were performed 24 h after transfection.

Tumor samples, RNA isolation, cDNA synthesis and quantitative-PCR

Total RNA was isolated from middle- to high-grade mammary carcinoma samples (TNM staging), and ErbB2 reactivity was defined by HercepTest (Dako North America Inc.) following the manufacturer's guidelines. Control RNA was derived from adjacent non-tumor tissue. Tissues were homogenized in phenol/guanidinium-isothiocyanat buffer (PEQLAB Biotechnologie), and poly A^+ mRNA was extracted from total RNA (Oligotex® poly-A^+ mRNA extraction kit, Qiagen). Poly A^+ mRNA was quantitatively reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) primed by Random Hexamers (Roche). Transcript levels were determined by real-time PCR using TaqMan® MGB (Applied Biosystems) chemistry on an Applied Biosystems 7500 Fast Real-Time PCR System. GAPDH expression was monitored as endogenous control.

Immunoprecipitation and in vitro kinase assay

Immunoprecipitations were performed as described [46]. Briefly, transiently transfected cells were lysed in lysis buffer A [120 mM NaCl, 50 mM Tris pH 7.5, 1% NP-40, 5 mM dithiothreitol (DTT), 200 µM vanadate, 10 mM pyrophosphate, 25 mM NaF, 1% aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysates were precleared with Pansorbin (Calbiochem, Germany) and incubated with anti-Syk mAb 4D10. Immunocomplexes were bound to Protein G-Sepharose (Sigma–Aldrich) and subsequently washed three times with TNE (140 mmol/l NaCl, 50 mmol/l Tris-HCl pH 8.0, 5 mmol/l EDTA) supplemented with 1% Nonidet P-40 (Roche Diagnostics, Mannheim) and twice with TNE. For subsequent kinase reactions, immunocomplexes were additionally washed with kinase buffer (50 mmol/l Tris-HCl pH 7.5, 8 mmol/l $MgCl_2$, 2 mmol/l $MnCl_2$, 1 mmol/l DTT).

Immunoprecipitates were resolved by SDS-PAGE, transferred to PVDF membranes (Westran® CS, Schleicher & Schuell, Germany) and immunoblotted with appropriate primary antibodies followed by HRP-conjugated secondary reagents. Proteins were visualized by chemoluminiscence,

using SuperSignal West® Pico Substrates (Pierce Biotechnology, Rockford, IL). Syk protein levels were quantified by Western blotting with (^{35}S)-coupled secondary antibodies and autoradiography.

For in vitro kinase assays, cells were starved in DMEM/0.5% FCS for 12 h and either directly lysed or re-plated for 2 h on Petri dishes coated with 125 µg/ml collagen I (BD Biosciences, San Diego, CA) or PBS/5% BSA (Carl Roth, Germany). Kinase reactions were performed for 20 min at 30°C in 50 µl kinase buffer supplemented with 10 µCi of [γ-^{32}P]-ATP and 2 µg recombinant GST:Igα-ITAM as a Syk substrate [46]. Reactions were terminated by mixing with Laemmli-sample buffer and boiling. Samples were resolved by 7.5% SDS-PAGE and autoradiographed. Kinase activity was quantified using the Lumianalyst Image Analysis Software.

Immunofluorescence staining

Murine tissues samples were dissected from adult female *Syk*-Cre/+ //*R26R*-YFP/YFP mice, fixed in 4% PFA for 4 h at RT, dehydrated via a sucrose gradient and embedded in Tissue Tek® O.C.T. compound (Sakura Finetek Europe, NL) and shock-frozen in isopentane at −80°C.

Then 10 µm cryosections were prepared, fixed in methanole, incubated in PermBlock solution [PBS, 1% (v/v) BSA, 0.5% (v/v) Tween], blocked in 90% goat serum/10% mouse serum and subsequently stained for E-cadherin and YFP using the rat anti-mouse E-cadherin antibody DECMA-1 (ascites fluid, Sigma-Aldrich) and rabbit anti-GFP antibody (Abcam plc UK) in conjunction with anti-rat AlexaFluor-568 and anti-rabbit AlexaFluor-488 secondary antibodies (Molecular Probes, Oregon). Sections were mounted in Mowiol and analyzed by confocal microscopy (LSM 510 Meta, Zeiss).

Migration and wounding assays

HC11 cells were serum-starved 24 h prior to the assays. Then 3.5×10^4 starved cells were plated on 8-µm-pore transwell filters (Corning Costar, Germany) and allowed to migrate towards fibroblast-conditioned medium (0.5% NCS) for 12 h. HB2 cells were switched to starvation medium (0.5% FCS) 24 h following siRNA transfection. After 12 h, 7.5×10^4 cells were plated on 8-µm-pore transwell filters and allowed to migrate towards fibroblast-conditioned culture medium (0.5% FCS) for 18 h. T47D cells were serum-starved 24 h following siRNA transfections. After 12 h, 5×10^4 cells were plated on 8-µm transwell filters coated with 15 mg/ml collagen I and allowed to migrate towards fibroblast-conditioned culture medium supplemented with 50 ng/ml EGF for 24 h.

Twenty-four hours after RNAi transfections, 7.5×10^4 MDA-MB-468 cells were plated on 8-µm-pore transwell filters and allowed to migrate towards fibroblast-conditioned culture medium (10% FCS) for 36 h.

After migration, cells were fixed in 4% PFA, non-migrated cells were removed with cotton swaps, and migrated cells were stained with 2 µg/ml of Hoechst dye. Cell numbers were determined by epifluorescence microscopy using the Image J software (NIH, Bethesda, MD).

5×10^6 serum-starved Cos1 cells, transiently transfected with pIRES-hrGFP-Syk-WT, pIRES-hrGFP1a-SykB or pIRES-hrGFP1a empty vector (Stratagene, USA), were seeded on non-transparent 8-µm Falcon HTS FluoroBlok™ Inserts. After 18 h migration towards fibroblast-conditioned medium, cells were stained with Hoechst dye, and green fluorescence on either side of the filters was determined at 8-bit depth, normalized for cell density (nuclei) and classified as follows: channels 0–59 were considered background, channels 60–130 originated from weak expression, channels 131–210 from intermediate and channels 211–255 from strong expression. Data were analyzed using the Image J software package.

For scratch wound closure assays, transiently transfected Cos1 cells were re-plated on collagen I-coated tissue culture dishes. Near confluent cells were treated with 10 µg/ml mitomycin C, and a scratch wound was introduced into the cell monolayer. Wound closure was monitored over at least 36 h by time-lapse life cell microscopy. Image analysis was done using the Image-Pro Plus 5.0 Software (Media Cybergenetics, MD). Statistical analysis was done using the Student's t test, and p-values of <0.05 were considered significant.

Results

Syk is selectively expressed in different murine epithelia

Syk expression has been demonstrated in various human epithelia, such as the ductal epithelium of the mammary gland by in situ RNA hybridization and immunohistochemistry [10, 26, 27] and in human lung and stomach epithelium by immunohistochemistry [28, 29]. To undertake a more systematic analysis of epithelial Syk expression in the mouse, we used a double transgenic mouse line that allows genetic tracing of *Syk* promotor activity in vivo and identifies cells with present or previous *Syk* promotor activity by YFP expression [30]. In the *Syk*-Cre$^{\Delta}$ mice, Cre-recombinase is expressed under the control of the endogenous *Syk* promotor. Cre-activity and hence *Syk* promotor activity can be detected by virtue of a YFP reporter allele, which is transcriptionally silenced by a loxP flanked *pgk*-neo cassette [31]. This approach harbors

significant advantages; first, it does not depend on the availability of murine Syk-specific antibodies suited for histochemistry. Second, the expression strength of the reporter is independent of the signal, which caused its activation, and third, activation of the reporter gene is accomplished by the integrated promotor activity over time; consequently, also small expression levels are faithfully detected.

We prepared cryosections from mammary gland tissue of *Syk*-Cre$^{\Delta}$/+ //*R26R*-YFP/YFP double transgenic mice and visualized present and previous *Syk* promotor activity by staining for YFP. Epithelial cells surrounding the mammary ducts were defined by E-cadherin expression. The *Syk* promotor was active in epithelial cells lining the mammary ducts and in myoepithelial cells surrounding the ductal epithelium. In addition, isolated cells of presumably hematopoietic origin, scattered within the surrounding fatty tissue, also stained positive (Fig. 1a–d). Identically stained mammary sections from *Syk* +/+ //*R26R*-YFP/YFP single transgenic reporter mice served as a negative control and displayed no staining for YFP (Fig. 1e–g). Moreover, we readily detected Syk expression in the stratified epithelium of the skin (Fig. 1h–j). In contrast, we could neither observe YFP staining in the columnar epithelium of the intestinum (supplementary Fig. 1a–c) nor in the cuboidal epithelium of the lung alveoli (supplementary Fig. 1d, e), demonstrating an unexpected selectivity of epithelial Syk promotor activity in the mouse.

In mammary carcinoma samples relative message amounts of SYK and DDR1 expression are shifted towards DDR1

Several studies have addressed changes in expression of primarily Syk, but also DDR1, during mammary tumor formation. Syk expression has been demonstrated to be lost during progression of a variety of epithelial tumor types [4]. However, the relative change in expression of both genes in the same sample has so far not been assessed. We performed q-RT–PCR on eight independent mammary tumor samples (medium to high grade carcinomas, Fig. 2c) and determined the change in DDR1 and Syk expression compared to samples of adjacent healthy breast tissue of the same individuals (Fig. 2a). Our data recapitulated the reported strong reduction in Syk expression in all eight samples. Relative changes in DDR1 expression were significantly less dramatic and entailed a small to moderate reduction in five cases, an increase in two cases and no change in one case. However, with the more than tenfold decrease in Syk expression levels, the mild absolute changes in DDR expression translate into a 4.7–1,600 fold increase in DDR1/Syk mRNA ratios in tumor tissue (Fig. 2b), i.e. a strong shift in favor of DDR1 expression (Fig. 2b).

Syk inhibits migration of Cos1 cells in a transwell model and a scratch wound closure assay

For an initial characterization of possible Syk functions in adherent cells, we expressed Syk in Cos1 and 293T cells. Both cell lines express low levels of endogenous Syk as compared to fresh murine bone marrow preparations. The endogenous Syk protein level in 293T cells is about three to five times higher than in Cos1 cells (Fig. 3a). It is important to note that during the subsequently reported transfection experiments, total Syk protein levels in transfected cells were at no time higher than endogenous Syk levels in bone marrow or epithelial cell lines like MDA-MB-468.

To test a possible function of Syk on cell migration, we transiently transfected Cos1 cells with a bi-cistronic vector encoding the renilla green fluorescent protein (rGFP) either alone or together with wild-type Syk (rGFP-SykWT) or SykB (rGFP-SykB). In the bi-cistronic vector, the upstream transcriptional entity rGFP is separated from the Syk cDNA by an IRES element, and both proteins are translated from the same mRNA transcript. To demonstrate the coupling of rGFP and Syk expression, we sorted transiently transfected Cos1 cells according to their GFP expression into a negative, low, medium or high expression population (Fig. 3b). We tested Syk kinase activity and Syk protein levels in the different populations (Fig. 3c), which correlated well with GFP expression. We therefore concluded that Syk protein levels could be inferred from the intensity of the green fluorescence.

We next tested cells with different Syk expression levels, as indicated by their different green fluorescence levels, in a chemotaxis-induced mobility assay. Unsorted, transiently transfected Cos1 bulk populations were seeded on optically dense filters with 8-μm pores, and their migration towards fibroblast-conditioned media was analyzed. As all cells migrated under identical conditions through the same filter, clonal variations because of selection of stable lines were excluded (a representative example of two filters is shown in Fig. 3d). In cells expressing only rGFP, 79% of the weakly expressing cells had migrated to the bottom of the filter, whereas only 72% of the intermediate and 60% of the strongly GFP-positive cells were found within the migrated population (Fig. 3e). These data revealed a mild migration inhibition caused by increasing rGFP expression levels. In contrast, SykWT expression caused a significant migration inhibition by far exceeding the effect attributable to rGFP. Only 46% of the weakly, 30% of the intermediately and 14% of the strongly Syk/rGFP expressing cells were found within the migrated population (Fig. 3d, e). Compared to SykWT, in this cell type SykB caused a less pronounced, but still significant, migration inhibition, with 70% of the weakly, 63% of the

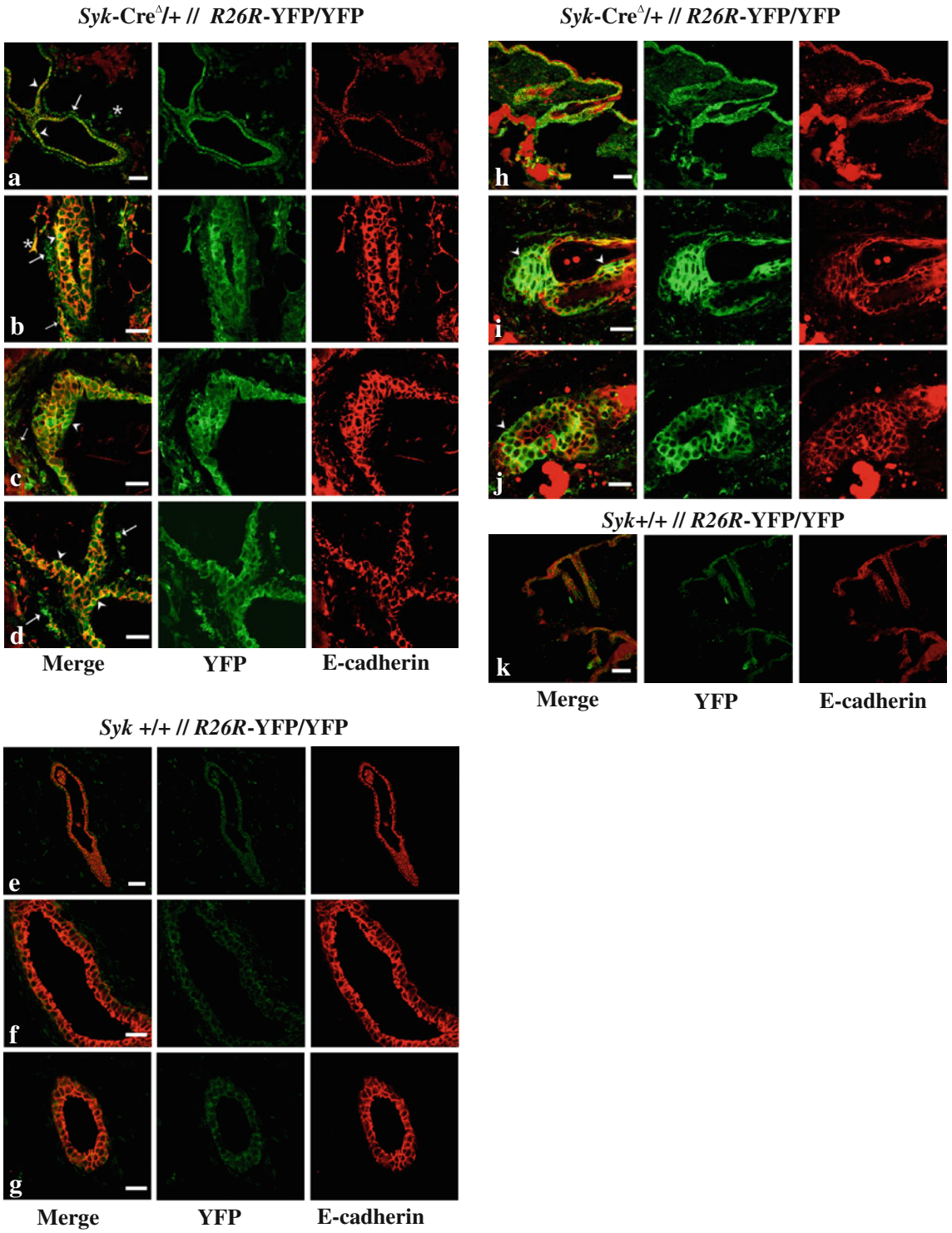
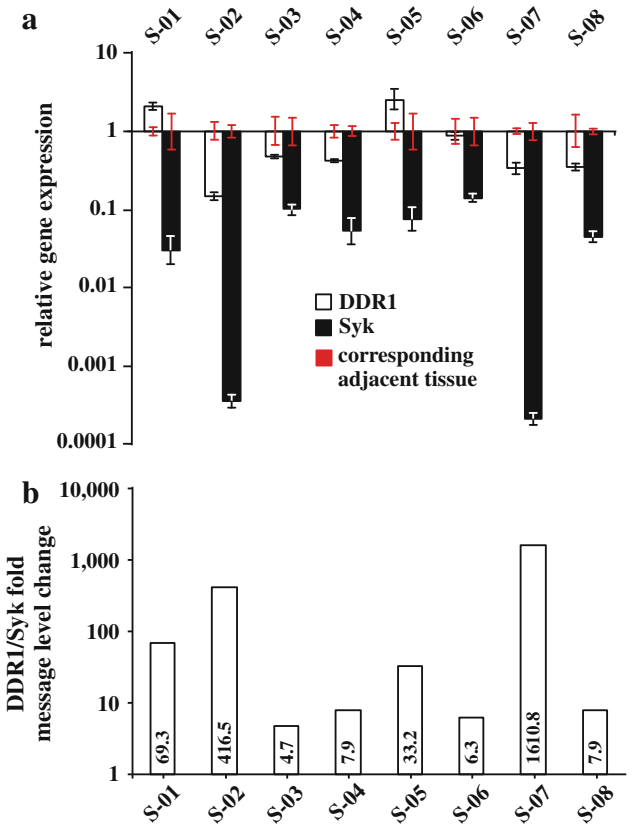


Fig. 1 Syk is expressed in murine breast and skin epithelium. To genetically trace Syk expression in different murine epithelia, cryosections of tissue samples derived from *Syk*-Cre$^{\Delta}$/+ //*R26R*-YFP/YFP double transgenic mice were prepared and counterstained for YFP (Alexa 488, *green*) and E-cadherin (Alexa 568, *red*). **a**–**d** Syk promotor activity, indicated by YFP staining, was detected in mammary ductal epithelial cells (*arrowheads*) and the surrounding myoepithelium (*arrows*), as well as in scattered cells within the surrounding fatty tissue (*asterisk*). **e–g** Cryosections from a single-transgenic *R26R*-YFP/YFP mouse served as a control and showed no YFP staining. **h–j** Syk promotor activity was also detectable in the stratified epithelium of the skin (*arrowheads*). **k** Corresponding sections from a single-transgenic *R26R*-YFP/YFP mouse served as a negative control for skin tissue. Bars correspond to 50 μm (**a**, **e**, **h**, **k**) or 20 μm (**b**, **c**, **d**, **i**, **j**)



c

Sample / Stage	S-01	S-02	S-03	S-04	S-05	S-06	S-07	S-08
T/N	T3,N0	T2,N2	T1,N2	na	T3,N0	na	T2,N1	T2,N0
HER2	+1	+3	0	0	0	2	+3	0

Fig. 2 Differential changes of Syk and DDR1 mRNA levels in human mammary carcinomas result in a relative shift towards DDR1 message. **a** Transcript levels of DDR1 and Syk in eight independent human mammary carcinoma samples and matched adjacent control breast tissues were determined by quantitative real-time PCR relative to GAPDH expression. Each qPCR reaction was performed in triplicate, and expression in the healthy control tissue was normalized to 1. *Error bars* corresponding to control tissue are indicated in *red*. DDR1 and Syk expression levels in the carcinoma tissues are depicted as fold changes in mRNA expression levels compared to the matched control tissues (ΔΔCt). *Black error bars* indicate the range of possible RQ values defined by the standard error of the ΔΔCts, again of triplicate measurements. **b** The ratio of the DDR1/Syk message level change in tumor tissues is depicted as *bars*. *Numbers inside the bars* denote the fold DDR1/Syk message level change in breast tumor tissue. **c** Staging of tumor samples (*T* tumor size; *N* involved regional lymph nodes according to TNM classification; *HER2* ErbB2 reactivity as defined by HercepTest guidelines). *na* Not available

intermediately and 44% of the strongly expressing cells being found at the bottom of the filter.

We next analyzed the capacity of Syk to inhibit spontaneous migration into a scratch wound. Following transient transfection of Cos1 cells with the above-described rGFP or rGFP-SykWT expression vectors, a scratch wound was introduced into a confluent monolayer, and migration was monitored by life cell microscopy. We traced the trajectories of ten cells, each displaying intermediate or high rGFP or rGFP-SykWT expression, and determined the accumulative migrational distance (Fig. 3f). We detected no statistically significant difference for either low or intermediate rGFP- or rGFP-SykWT-expressing cells. In contrast, strongly rGFP-SykWT-expressing cells migrated significantly more slowly than control cells expressing rGFP only.

DDR1 interacts with Syk and stimulates Syk kinase activity

Syk has been reported to bind to the discoidin domain receptor (DDR)1 in HB-2 and MCF-7 epithelial cell lines [8]. We therefore tested a potential regulation of Syk kinase through DDR1. To quantify Syk kinase activation in transiently transfected cells, we measured in vitro autophosphorylation of immunopurified Syk, as well as transphosphorylation of a GST fusion protein containing the Igα intracellular tail. Albeit not expressed in epithelial cells, Igα is a well-established, excellent Syk target and thus provides a well-suited surrogate substrate [32]. Considering a possible collagen I dependency of Syk activity, we determined Syk activation following replating of the transfected cells on either collagen I or heat-denatured BSA (negative control).

In Syk immunoprecipitates of empty vector-transfected 293T cells, we detected a low-level in vitro kinase activity (Fig. 4a, top panel), which likely originates from endogenous Syk protein (Figs. 3a, 4a). Moderate exogenous Syk expression resulted in a four to fivefold enhanced Syk activity, which was further stimulated 1.3 fold by plating on collagen I.

Remarkably, co-expression of DDR1 caused an additional doubling in Syk kinase activity, revealing a robust regulation of Syk activity by DDR1 (Fig. 4a, bottom panel). DDR1 was detected in Syk immunoprecipitates, confirming the interaction between both proteins (Fig. 4b, top panel), and was phosphorylated on tyrosine upon plating on collagen I (Fig. 4b, middle panel), indicating activation of the DDR1 receptor after collagen binding. In contrast, neither the interaction of DDR1 and Syk, nor the activation of Syk following DDR1 binding was collagen dependent.

In Cos1 cells, which only express traces of endogenous Syk protein (Fig. 3a), we did not detect endogenous kinase activity after Syk precipitation. However, exogenously expressed Syk also showed a significant, 3.5-fold increase in Syk kinase activity upon co-expression of DDR1 (Fig. 4c). In addition, both proteins colocalized in Cos1 cells (not shown). Concomitantly with the robust

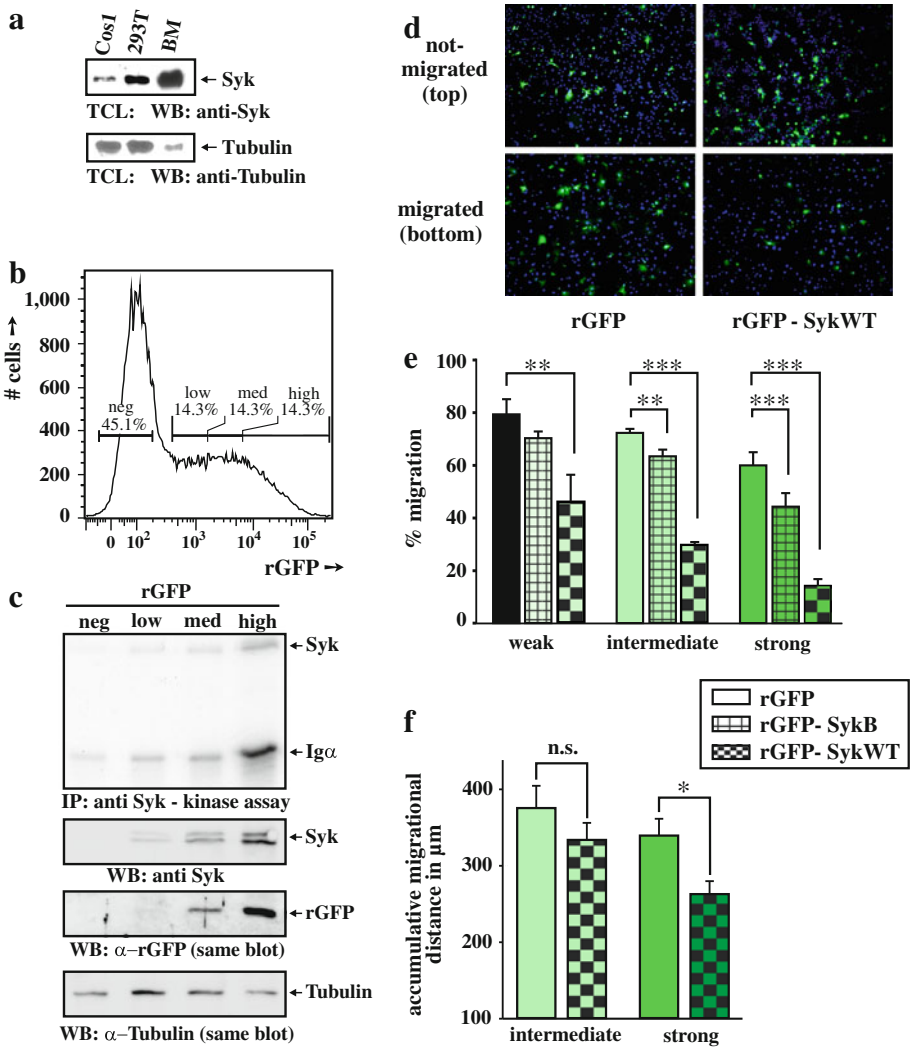


Fig. 3 Syk mediates migration inhibition in Cos1 cells. **a** Total cell lysates (TCL) of the indicated cell lines or freshly harvested bone marrow cells were subjected to Western blotting and probed either with an anti-Syk antibody or with an anti-Tubulin antibody as a loading control. **b** Cos1 cells were transiently transfected with the expression plasmids pIRES-rGFP-SykWT and sorted for GFP signal intensity on a FACSAria [negative (neg), low (low), medium (med) and high (high)]. **c** Sorted cells (**b**) were harvested and subjected to an in vitro kinase assay in the presence of a GST-Igα fusion protein (*top panel*). Total cell lysates (TCL) were subjected to Western blotting (WB) and probed with the indicated antibodies. **d** Syk inhibits chemotactic migration in Cos1 cells. Cos1 cells were transiently transfected with the expression plasmids pIRES-rGFP-SykWT (rGFP–SykWT), pIRES-rGFP-SykB (rGFP–SykB) or pIRES-rGFP (rGFP) and after 36 h replated on non-translucent porous filters. Migration towards fibroblast-conditioned medium was determined over a total period of 18 h. *Top* and *bottom* of a representative filter are shown. **e** Quantitative evaluation of (**d**): Syk expression was classified according to the green fluorescence of the rGFP-protein into low, intermediate and strongly expressing cells. Events in each class were normalized for the total number of cells, as determined by counting of Hoechst 33258-stained nuclei. Bars depict the percentage of migrated events of each class and represent the average of four independent experiments. **f** Syk suppresses spontaneous migration of Cos1 cells into a scratch wound. Cos1 cells were transiently transfected as described in (**d**), mitogenically arrested by mitomycin C treatment, replated on collagen I, and a scratch wound was introduced into the near-confluent monolayer. Migration of cells into the scratch wound was monitored using time lapse microscopy. Of each group the trajectories of ten intermediate or strong fluorescing cells were tracked. The bar diagram depicts the average cumulative migrational distance in µm. **e**, **f** *Error bars* indicate the SEM; statistically significant differences (*t* test) are indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *ns* not significant

enzymatic activation of Syk in the presence of DDR1, Syk phosphorylation on tyrosine was strongly enhanced. We wondered, if the activation of Syk in the presence of DDR1 involved phosphorylation of Syk by DDR1 kinase. However, Syk activation and phosphorylation were independent of DDR1 kinase activity, as they were also elicited by a

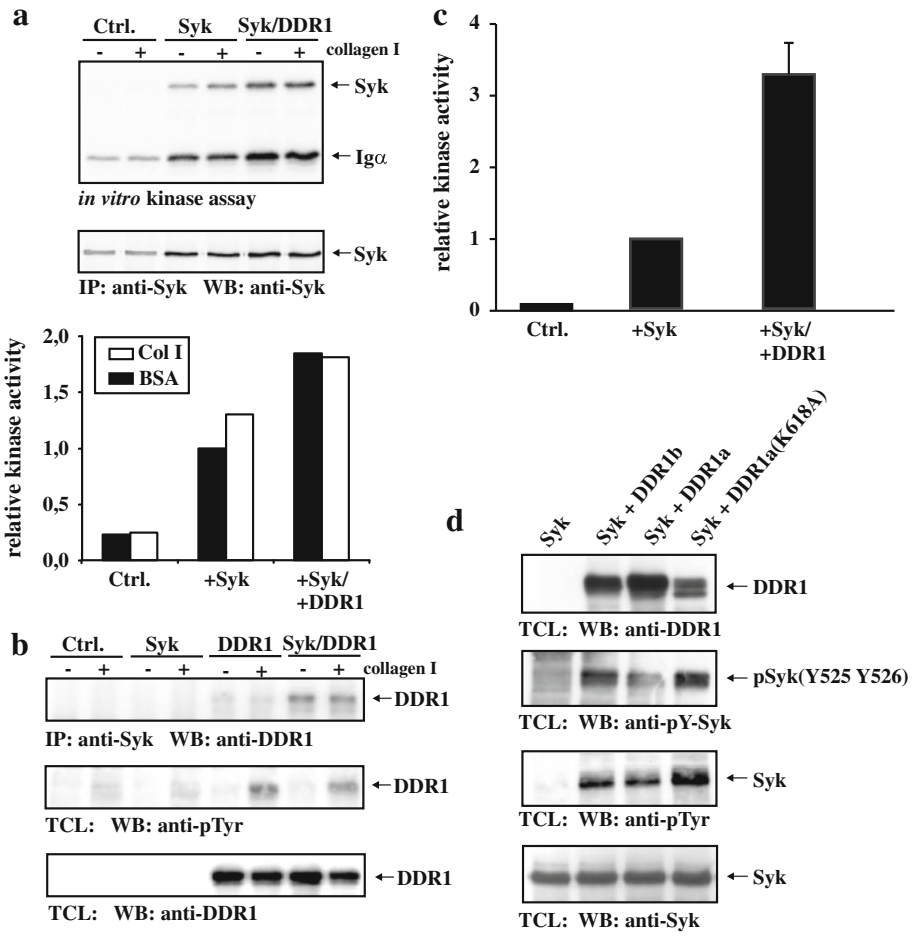


Fig. 4 DDR1 interacts with Syk and stimulates Syk kinase activity in 293 and Cos1 cells. **a** 293T cells were transfected with expression constructs encoding the indicated proteins (control, empty vector) and 48 h later replated on Petri dishes coated with either collagen (+) or heat-denatured BSA (−). After 2 h, cells were harvested and subjected to an in vitro kinase assay in the presence of a GST-Igα fusion protein (*top panel*). Relative Syk kinase activity towards Igα was determined densitometrically and normalized for Syk protein levels (*bottom panel*). Shown is one representative experiment of four; activity of Syk-transfected cells on BSA was defined as 1 (*bar diagram*, *bottom*). **b** Syk and DDR1 coprecipitate in 293T cells. Cells were transfected and plated as described in (**a**). Either total cell lysates (TCL) or immunoprecipitated (IP) Syk protein was subjected to Western blotting (WB) and probed with the indicated antibodies. All results are representative of at least three repeats with identical outcome. **c** DDR1 activates Syk kinase in Cos1 cells. Cos1 cells were transfected with the indicated expression constructs or empty vector (control) and harvested for an in vitro kinase assay after 48 h. Kinase activity towards a GST-Igα fusion protein was determined in vitro. Activity of Syk-transfected cells was defined as 1. **d** Cells were transfected with an expression construct encoding Syk and the indicated forms of DDR1, lysed after 48 h and immunoblotted for the denoted proteins

kinase inactive version of DDR1a (DDR1a(K618A)) (Fig. 4d). Taken together, our findings demonstrate an interaction between Syk and DDR1 in adherent cells resulting in a robust activation of Syk kinase activity.

Syk-mediated inhibition of chemotactic migration of HC11 epithelial cells is blocked by DDR1 receptors

Next we tested whether Syk also inhibits chemotactic migration in epithelial cells and possible consequences of the presence of DDR1 on epithelial cell mobility. Therefore, we turned to the non-transformed mouse epithelial cell line HC11. Using recombinant retroviruses, we generated HC11 populations stably expressing SykWT, SykB or the kinase-deficient mutant Syk(K402R) (Fig. 5a, left panel). Expression of the corresponding Syk variants was verified by Western blot analysis and revealed physiological Syk expression levels, comparable to the endogenous Syk levels in the epithelial cell lines HB2, T47D and MDA-MB-486, which was also the case for HC11 cells additionally expressing DDR1.

We then tested the capacity of the populations to migrate in a transwell filter system (Fig. 5b–d). As shown before for Cos1 cells, HC11 cells expressing SykWT displayed a significant reduction of cell migration by 45% compared to control cells infected with parental virus.

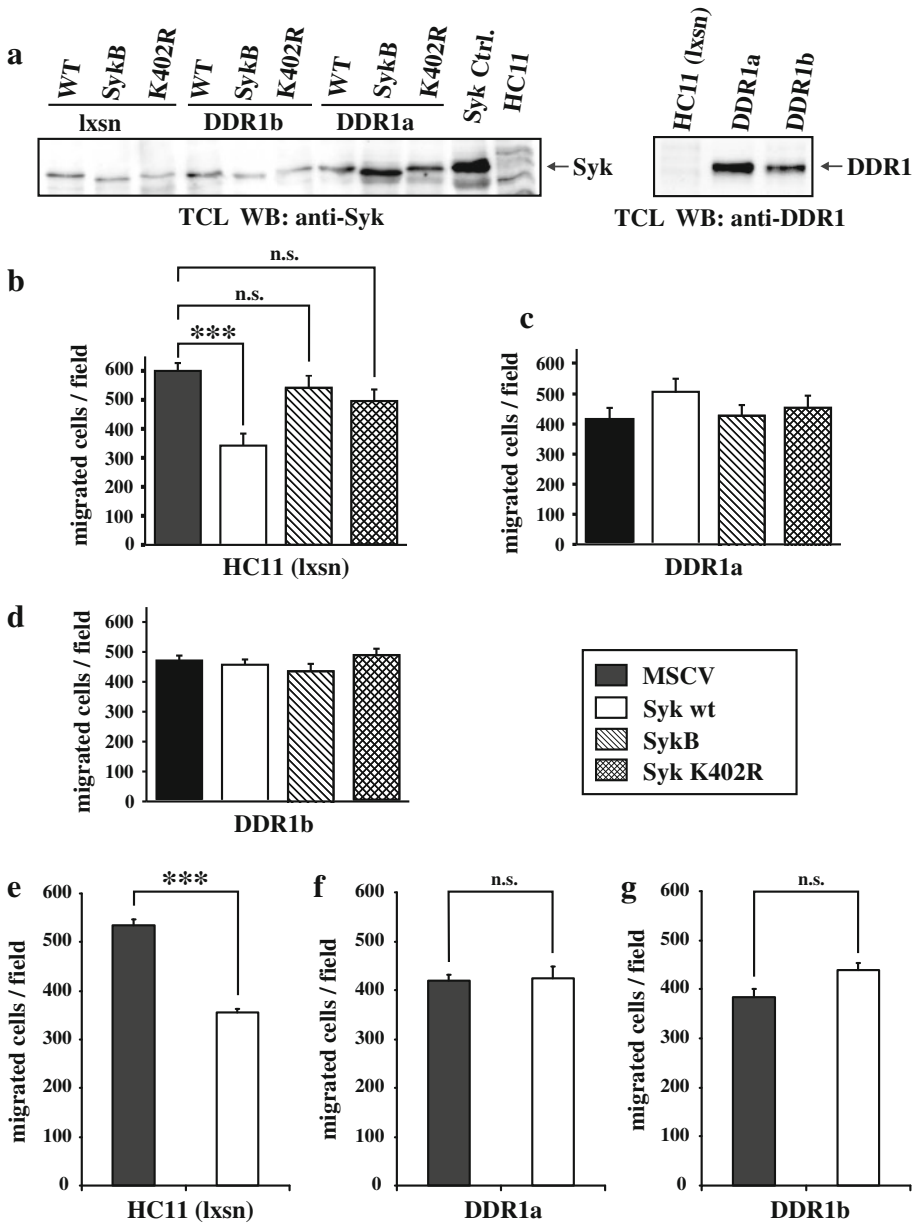


Fig. 5 Syk causes migration inhibition in HC11 murine breast epithelial cells. **a** HC11 cells were infected with recombinant retroviruses stably transducing DDR1a, DDR1b or control virus (lxsn) (*right panel*). Subsequently, SykWT, SykB or the kinase dead mutant Syk(K402R) were expressed in each of the three groups by superinfection with recombinant replication-defective retrovirus. Successful transduction was visualized by Western blotting of total cell lysates (*left panel*). **b** Syk suppresses fibroblast-induced chemotaxis of HC11 epithelial cells. Stably transduced HC11 bulks were seeded onto porous filters and allowed to migrate towards fibroblast-conditioned medium. Migrated cells were stained with Hoechst 33258, nuclei were visualized by epifluorescence microscopy and counted. (**c, d**) DDR1 receptors block the migration inhibition by Syk in epithelial cells. HC11 bulks co-expressing the indicated variants of Syk and DDR1a (**c**) or DDR1b (**d**) were analyzed as described in (**b**). **e–g** Syk-mediated migration inhibition is independent of collagen I. The indicated HC11 cell populations were seeded on collagen I-coated transwell filters and analyzed as described in (**b–d**). All experiments were repeated at least three times in triplicate. For each filter, the cell number in 16 non-overlapping view fields was enumerated. *Error bars* SEM, statistically significant differences (*t* test) are indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *ns* not significant

HC11 cells expressing SykB or Syk(K402R) showed a minor migration reduction of 10 and 17%, respectively, which was not significant.

To analyze the potential of DDR1 receptors to influence cell motility in the presence of Syk, HC11 populations expressing additionally either DDR1a or DDR1b were subjected to the transwell chamber assay (Fig. 5a, right panel). Notably, Syk-mediated migration inhibition was no longer observed in the presence of either DDR1 isoform (Fig. 5c, d). We addressed a possible collagen dependence

by repeating HC11 migration assays on collagen I-coated transwell filters. Syk also significantly inhibited the migration of HC11 cells through collagen I-coated transwell filters by 34% (Fig. 5e). Again, this inhibition was blocked in the presence of DDR1a or DDR1b (Fig. 5f, g). Hence, in keeping with our previous notion of a collagen-independent interaction between Syk and DDR1, we confirmed the anti-migratory effect of Syk in HC11 epithelial cells on non-coated and collagen I-coated filters and demonstrated its blockade in the presence of DDR1.

Ablation of DDR1 reduces cell motility in various breast epithelial cell lines

To provide further support for a pro-migratory role of DDR1, we knocked down DDR1 expression in the well-established breast epithelial cell lines T47D, HB2 and MDA-MB-468, all of which endogenously express both proteins, Syk and DDR1.

As demonstrated by Western blot analysis, we achieved a close to complete deletion of the DDR1 protein 24 h after siRNA transfection, which was stable for at least 48 h (Fig. 6a–c). Similar results were obtained with two non-related siRNAs; one example is shown. As shown in Fig. 6d, siRNA-mediated knockdown of DDR1 did not affect Syk protein levels compared to control cells. Again, we tested the migration of siRNA-transfected cells in chemokine-driven transwell migration assays and detected a robust reduction of cell migration upon DDR1 silencing in all cell lines analyzed (23% for T47D, 27% for HB2 and 57% for MDA-MB-468) (Fig. 6e–g). This finding suggests that the DDR1 receptor fulfills a pro-migratory role in epithelial cells. Interestingly, after DDR1 knockdown, MDA-MB-468 cells, which express the highest endogenous Syk level, displayed the lowest migratory activity, suggesting that the migratory capacity of the different epithelial lines correlated inversely with the endogenous Syk expression level (Fig. 6d–g).

Discussion

The non-receptor tyrosine kinase Syk has predominantly been studied in hematopoietic cells, where it acts as an essential signal transducer downstream of ITAM-containing immune recognition receptors. More recently, the observation that Syk is expressed in mammary epithelium and low tumorigenic breast cancer cell lines, whereas it is not detectable in metastatic breast cancer samples, has sparked considerable interest [4]. Whereas expression of Syk in the human mammary gland, gastric and lung epithelium has been demonstrated by immunohistochemistry [27], a more systematic analysis of Syk expression in mouse epithelia was so far not available. We tested several commercially available anti-human Syk antibodies and found them unsuitable to detect the murine protein in immunohistochemistry. We therefore used genetic tracing to identify Syk-expressing cells with high sensitivity and cellular resolution. We found Syk expressed in different cell types within the mammary gland, including the ductal epithelial cells and myoepithelial cells surrounding the epithelial lining of the milk ducts. Furthermore, we noted scattered Syk-positive cells in the surrounding fatty tissue, which were of irregular shape and likely of hematopoietic origin. Under the same conditions, we detected Syk expression in the stratified epithelium of the skin, whereas it was absent from the columnar epithelium of the small intestine and the squamous epithelium of the lung. The epithelial lining of the gastrointestinal and respiratory tract is of endodermal origin, whereas the epidermis and its appendages arise from the embryonic ectoderm. We did not detect Syk expression in other ectodermal derivatives, like the central and peripheral nervous system. Furthermore, we first noted Syk expression in the skin around day E16.5 of development, when in the mouse the primary mammary bud has already formed. This finding makes it unlikely that the signal we detected in skin and mammary gland is due to Syk expression in a common progenitor cell. However, because of the genetic approach we took, our analysis does not distinguish between ongoing and previous Syk expression. Further studies will therefore have to attempt direct detection of Syk protein in adult mammary tissue.

Based on the apparent loss of expression during mammary tumor progression, Syk has been classified as a tumor suppressor; however, the molecular basis remains unclear. Conflicting reports on the function of Syk in cell migration attributed inhibitory as well as promigratory effects to this kinase. In squamous cell carcinomas, Syk was shown to positively regulate cell motility [33]. Our analysis revealed a prominent Syk-mediated anti-migratory effect on chemotaxis-induced and spontaneous migration of adherent cells that was dependent on Syk kinase activity. The variant SykB showed a reduced migration inhibitory effect in Cos1 cells and was ineffective in HC11 epithelial cells, pointing either toward different intracellular interaction partners or a different subcellular localization in these two cell types. SykB has previously been shown to be unable to suppress breast cancer invasiveness and to be exclusively localized to the cytoplasm, whereas Syk is also found in the nucleus [26]. In concordance with our observations, Syk has been reported by several studies to inhibit chemoinvasion through matrigel [7, 11, 26], while antisense-mediated knockdown increased cell mobility [34]. The exact mechanism underlying Syk-mediated migration inhibition remains to be elucidated. In MDA-MB-231 cells, Syk has been suggested to reduce motility by suppression

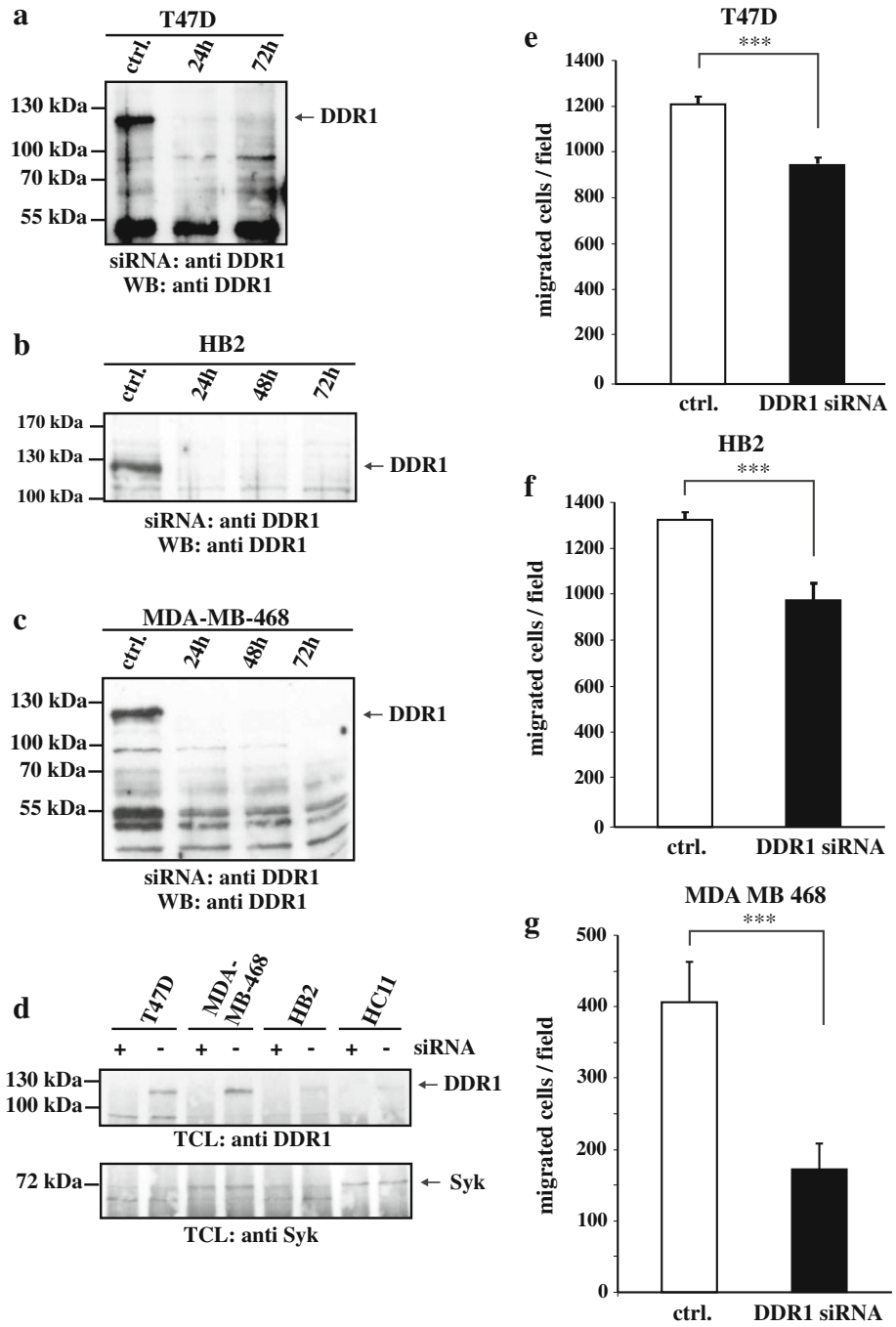


Fig. 6 Ablation of DDR1 in different breast epithelial cell lines results in migration inhibition. **a–c** T47D, HB2 and MDA-MB-468 breast epithelial cells were transfected with DDR1 siRNA. siRNA-mediated DDR1 knockdown was verified by Western blot analysis of total cell lysates. **d** DDR1 was specifically knocked down following siRNA transfection, whereas Syk expression levels remained stable. **e–g** DDR1 knockdown cells were seeded on transwell filters and allowed to migrate towards fibroblast-conditioned medium. Migrated cells were stained with Hoechst 33258; nuclei were visualized by epifluorescence microscopy and counted. *Error bars* SEM, statistically significant differences (*t* test) are indicated, $***p < 0.001$

of PI-3-kinase and uPA secretion [34]. More recently, Syk has been reported to inhibit migration of MDA-MB-231 cells and to promote cell-cell contact formation by promoting the localization of vinculin and cortactin to cell-cell contact sites [35].

In the course of this study, we noted a profound positive effect of Syk on cell adhesion and spreading on collagen that was accompanied by a moderate, but lasting, increase in Syk kinase activity (BN not shown). In platelets, Syk contributes through "inside out" signaling mechanisms to adhesion and spreading via the collagen receptor $\alpha 2\beta 1$ and the fibrinogen receptor $\alpha 2b\beta 3$ [36, 37]. Similarly, activation of Syk via $\beta 1$ integrins [8, 35] and subsequent "inside out" signaling could cause increased adhesion on collagen. In addition, Syk could directly act on adhesion complexes at cell junctions. A recent report described Syk localization at epithelial cell-cell contacts and Syk-mediated phosphorylation of E-cadherin. This resulted in an enhanced interaction with p120-catenin and strengthening of the adherence junctions [38]. Depletion of Syk in K-RAS-dependent epithelial cancer cell lines led to the loss of E-cadherin, reminiscent of epithelial-mesenchymal

transition (EMT) [39]. Taken together, there is strong evidence for an anti-migratory function of Syk in epithelial cells, which may involve increased adhesion to ECM components, direct enforcement of cell-cell adhesion, as well as changes in transcriptional profile and cytoskeletal architecture.

The receptor tyrosine kinase DDR1 functions in the regulation of cell adhesion and migration [12, 23, 40, 41]. In breast epithelial cells, constitutive interaction of DDR1 and Syk has been reported [8]. We investigated tyrosine phosphorylation and migration behavior of epithelial cells expressing both kinases. We also detected a collagen I-independent, constitutive interaction between Syk and DDR1. In addition, Syk kinase was activated in the presence of DDR1, and Syk auto-phosphorylation was prominently increased. Furthermore, the robust reduction in cell motility upon Syk-expression was no longer observed upon co-expression of DDR1 receptors. Reduced motility of HC11 cells expressing DDR1a or DDR1b likely reflected experimental or clonal variations, as siRNA-mediated knockdown of DDR1 in several different breast epithelial lines resulted in significantly reduced motility. In eight independent human mammary tumor samples analyzed, we found a strong reduction of Syk expression, whereas DDR1 expression was either weakly reduced or even moderately increased, shifting the ratio between Syk and DDR1 and in favor of DDR1 receptors.

We speculate, that DDR1-binding may not only result in Syk activation, but also Syk kinase activity might be redirected from pathways suppressing migration to pathways more compatible with transformation. Sequestration of Syk to specific subcellular compartments may be an important part of this action. In this respect, our findings are reminiscent of the interaction of the ITAM-containing protein C35 and Syk [42]. C35 is a short membrane-anchored protein that was identified on the basis of its strong upregulation in early and late stage breast carcinomas [43]. C35 binds and activates Syk, and promotes transformation and invasive behavior of epithelial cells [42]. Interestingly, expression of Syk-binding ITAM sequences per se in mammary epithelial cells can induce transformed behavior, which again is dependent on Syk kinase activity [44, 45]. Why Syk is lost later during tumor progression remains enigmatic; possibly its redirected activity is only needed initially during the transformation process.

In summary, we have demonstrated here that Syk promotor activity is detectable in mammary and stratified skin epithelium of the mouse and that Syk functions in the regulation of epithelial cell motility. Syk expression led to a significant decrease in cell migration; however, co-expression of the receptor tyrosine kinase DDR1 resulted in a strong activation of Syk, along with a blockade of Syk-mediated migration inhibition. Loss of Syk, deregulated expression of DDR1 or both may help epithelial cells to adopt a more mobile state. Intriguingly, both events have been associated with the malignant progression during breast cancer formation.

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