The Oncogenic Epidermal Growth Factor Receptor Variant *Xiphophorus* Melanoma Receptor Kinase Induces Motility in Melanocytes by Modulation of Focal Adhesions

Svenja Meierjohann,[1] Elisabeth Wende,[1] Anita Kraiss,[1] Claudia Wellbrock,[2] and Manfred Schartl[1]

[1]Department of Physiological Chemistry I, Biocenter, University of Wuerzburg, Wuerzburg, Germany and [2]Signal Transduction Team, Cancer Research UK Centre of Cell and Molecular Biology, The Institute of Cancer Research, London, United Kingdom

Abstract

One of the most prominent features of malignant melanoma is the fast generation of metastasizing cells, resulting in the poor prognosis of patients with this tumor type. For this process, cells must gain the ability to migrate. The oncogenic receptor Xmrk (*Xiphophorus* melanoma receptor kinase) from the *Xiphophorus* melanoma system is a mutationally activated version of the epidermal growth factor receptor that induces the malignant transformation of pigment cells. Here, we show that the activation of Xmrk leads to a clear increase of pigment cell motility in a fyn-dependent manner. Stimulation of Xmrk induces its interaction with the focal adhesion kinase (FAK) and the interaction of active, receptor-bound fyn with FAK. This results in changes in FAK activity and induces the modulation of stress fibers and focal adhesions. Overexpression of dominant-negative FAK shows that the activity of innate FAK and a receptor-induced focal adhesion turnover are a prerequisite for pigment cell migration. Our findings show that in our system, Xmrk is sufficient for the induction of pigment cell motility and underlines a role of the src family protein tyrosine kinase fyn in melanoma development and progression. (Cancer Res 2006; 66(6): 3145-52)

Introduction

Melanoma are among the most aggressive tumors in humans with a high metastatic potential. They arise from the transformation of melanocytes, a process that is dependent on both environmental and genetic risk factors. When melanocytes in the human skin become transformed, the cells start to proliferate in an uncontrolled manner (radial growth phase), lose contact inhibition, and start to migrate out of the epidermis (vertical growth phase).

Factors that permit melanoma cell migration include the enhanced expression of the growth factors transforming growth factor-α and heregulin/neuregulin, but also changes in integrin composition, e.g., induction of $\alpha_v\beta_3$ or $\alpha_2\beta_1$ expression (1–6). Both pathways can lead to activation of the focal adhesion kinase (FAK). In melanoma and other tumor types, increased FAK expression and phosphorylation has often been correlated with tumor progression and metastasis (7, 8).

A large fraction of FAK is localized in focal contacts, and its activity is required for adhesion and migration (for review, see ref. 9). When migrating cells are visualized, a smaller part of their cell surface is in contact with the substratum compared with resting cells (10, 11). The FAK-dependent focal contact turnover is increased, as the cell has to attach and detach during the course of migration (12). Active FAK binds to src, usually leading to further FAK activation and the formation of a FAK-src signaling complex (12).

The importance of src in FAK signaling has been studied intensely, and most reports that connect src-related kinases to migration deal with the prototypic member of the protein family, namely src itself (12–14). In contrast, only few reports show interactions of FAK with the closely related protein kinase fyn. An interaction was either observed in neuronal cells (15–18) or in Chinese hamster ovary and corneal endothelial cells highly overexpressing recombinant FAK or fyn (19, 20). Although the kinase seems to play a critical role in tumor formation (21, 22), its role for tumor migration is only scarcely investigated (23, 24).

Fyn was identified as crucial downstream substrate in *Xiphophorus* melanoma receptor kinase (Xmrk)–induced melanocyte transformation. Xmrk-driven melanoma formation in *Xiphophorus* is a model for receptor tyrosine kinase (RTK)–induced tumorigenesis and a well-established model for the molecular analysis of the successive steps in melanoma development (25, 26). Overexpression and mutation of this variant of epidermal growth factor (EGF) receptor (EGFR) induces all molecular events to trigger melanoma formation in this model system (27–29). The Xmrk-induced melanomas are fast-growing, highly invasive tumors, suggesting that the receptor not only stimulates proliferation but also induces an increased migration of the pigment cells.

To analyze Xmrk-induced signaling that is relevant for melanoma formation, we have established a construct to get a system in which a chimeric receptor consisting of the extracellular part of the human EGFR and the intracellular part of Xmrk (HERmrk) is used in mouse melanocytes (Melan-A). The lack of internal mouse EGFR in these cells and the usage of human EGF for the stimulation of HERmrk excludes that the observed signaling was due to an intrinsic mouse receptor. When HERmrk is stably expressed in Melan-A, which normally only grow in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and cholera toxin, application of human EGF alone renders the cells independent of these factors and induces transforming intracellular events. Using this system, several Xmrk-dependent pathways resulting in stimulation of proliferation, protection from apoptosis, and interference with differentiation have been observed (29–31). The high homology between mammalian EGFR and Xmrk allows the interaction with all hitherto investigated interaction partners of Xmrk in fish and mouse cells (29). The tyrosine residues that serve

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as docking sites for signal transducers like Grb2, Shc, src kinases, and PLCγ are highly conserved between the species. Similarly, the signal-transducing proteins themselves are highly conserved between fish and mammals, especially in their respective SH2 domains. Xmrk is a very potent oncogene and it is not only able to transform fish cells, but also mammalian cells (29). For this reason, it can also serve as a model for RTK-driven tumorigenesis in mammalian melanocytes to gain a better understanding of the processes that lead to the neoplastic phenotype.

To find out whether Xmrk is also involved in melanoma cell migration and thus contributes to invasion, we first used the HERmrk mouse melanocyte system (Melan-A Hm). We show that Xmrk confers migratory activity to the otherwise scarcely motile melanocytes. We show that the src kinase fyn and its interaction with FAK are essential for motility and that fyn is involved in Xmrk-dependent focal adhesion turnover. In a second set of experiments, we could verify these results for PSM cells that are derived from Xmrk-overexpressing melanoma.

This attributes an important role to fyn for melanomagenesis, acting not only on mitogen-activated protein kinase–dependent proliferation and prevention of apoptosis as described before (31, 32) but also on FAK-dependent migration.

Materials and Methods

Cell culture. Mouse melanocytes [Melan-A (33) and Melan-A Hm cells (31), clones TPAVI and EGF IX/3] were cultured in DMEM, 10% FCS in the presence of cholera toxin (12 nmol/L), and TPA (200 nmol/L). The melanoma cell line PSM from *Xiphophorus* was cultured in F12, 10% FCS at 28°C as described previously (34, 35).

Cell lysis and Western blotting. The cells were starved for 24 hours with DMEM containing 2.5% dialyzed FCS. After stimulation with indicated concentrations and times, cells were rinsed twice with PBS and lysed in 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl$_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 200 μmol/L Na$_3$VO$_4$, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L NaF. Fifty micrograms of protein lysate were separated by SDS-PAGE and analyzed by Western blotting onto nitrocellulose. Membranes were blocked for 30 minutes with TBS [10 mmol/L Tris-HCl (pH 7.9) and 150 mmol/L NaCl], 0.1% Tween, and 1.5% bovine serum albumin (BSA) and were incubated overnight at 4°C with the first antibody. Horseradish peroxidase–coupled second antibodies were used for nonradioactive detection. Polyclonal anti-FAK (A-17) and anti-fyn (FYN3) were from Santa Cruz Biotechnology (Santa Cruz, CA). According to the manufacturer, the anti-FAK antibody recognizes a FAK epitope that is well conserved between the species and reportedly recognizes FAK from such distant organisms as mammals, chicken, and amphibians. Polyclonal anti-mrk recognizing the COOH-terminal part of Xmrk ("pep-mrk") was generated by Biogenes (Berlin, Germany). Polyclonal anti-p85 was from Transduction Laboratories. Monoclonal antiphosphotyrosine (PY20) was from BD Transduction Laboratories (BD Biosciences, CA). The secondary antibodies were conjugated with horseradish peroxidase and were directed against mouse (Pierce, Rockford, IL) or rabbit (Bio-Rad, Hercules, CA).

Immunoprecipitation. The cells were starved for 24 hours with DMEM containing 2.5% dialyzed FCS. After cell lysis, 400 μg of the whole cell lysate was diluted 1:1 in HNTG buffer [20 mmol/L HEPES (pH7.5), 150 mmol/L NaCl, 10% glycerol, 0.1% Triton X-100, 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 200 μmol/L Na$_3$VO$_4$]. Protein A Sepharose (25 μL, diluted 1:1 in HNTG buffer) and 1 μg of the respective antibody were added, and the sample was incubated overnight at 4°C. After washing with HNTG buffer, Laemmli buffer was added to the Sepharose beads. The sample was then separated by SDS-PAGE and a Western blot was done.

Immunofluorescence. Cells (2 × 10^5) were seeded on glass coverslips and starved for 24 hours in DMEM with 2.5% dialyzed FCS. After treatment with 20 μmol/L AG555, 20 μmol/L PP2, DMSO (as control), or 100 ng/mL EGF for

the indicated times, the cells were fixed for 5 minutes in methanol (−20°C) and permeabilized for 2 minutes in acetone (−20°C). The samples were then blocked for 20 minutes with PBS/1% BSA and incubated with anti-FAK antibody (1:100, Santa Cruz Biotechnology) for 1 hour. After three washing steps, the coverslips were incubated with the second antibody (CY3-conjugated goat anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour in the dark. After four more washing steps with PBS and H$_2$O, the coverslips were embedded in 4′,6-diamidino-2-phenylindole–containing mounting medium (Vectashield Linaris, Wertheim, Germany).

Phalloidin staining. Cells (2 × 10^5) were seeded on glass coverslips and starved for 24 hours in DMEM with 2.5% dialyzed FCS. After treatment with 20 μmol/L AG555 or 100 ng/mL EGF for the indicated times, the cells were fixed in 2% paraformaldehyde in PBS for 20 minutes. Paraformaldehyde was then quenched with 0.1 mol/L glycine in PBS for 2 minutes. After four washing steps with PBS and H$_2$O, the coverslips were incubated with 15 μg/mL FITC-conjugated phalloidin (Sigma, St. Louis, MO) for 40 minutes in the dark. Subsequently, the samples were washed and embedded in Vectashield mounting medium.

Transwell migration assay. Melan-A Hm cells (2.5 × 10^4) were serum starved in DMEM, 1% dialyzed FCS for 24 hours, and applied to the upper chamber of a transwell inlay (polycarbonate, 10 mm diameter, 8 μm pores; Nunc, Rochester, NY) in DMEM with 1% dialyzed FCS. In the initial experiment, 1 ng/mL to 10 μg/mL EGF were applied to the lower chamber. For further assays, 100 ng/mL EGF was used. Inhibitors were added to the upper chamber in the following concentrations: AG555, 20 μmol/L; PP2, 20 μmol/L; LY294002, 10 μmol/L. Cells without inhibitor treatment received the equivalent volume of DMSO. After 12 hours, the transwell assay was stopped. The cells on the upper side of the membrane were removed with a cell scraper before the membrane was fixed for 5 minutes in methanol and stained for 20 minutes with 2% crystal violet dissolved in 2% ethanol. The membranes were then washed with PBS and the number of cells on the lower side of the membrane was counted. The migration rate was determined either in absolute numbers or as fold migration compared with the untreated cells in case of the inhibitor experiments. For PSM cells, the transwell assay was done in a similar manner but with the following modifications: Migration was done on transwell inlays that were precoated with 10 μg/mL collagen I for 1 hour. As a chemoattractant, 10% FCS was added to the lower compartment. The migration assay was done at 28°C for 20 hours.

In a separate assay, either pRK5-GST-Xfyn2 and pRK5 control vector (36) or FRNK-pcDNA3 and pcDNA3 control vector were cotransfected on day 1 with pEGFP-N1 (2.5:1; Clontech, Palo Alto, CA) in Melan-A Hm cells with Gene Juice as described (Novagen, Madison, WI) or in PSM cells with 1 mg/mL polyethylenimine (DNA-polyethylenimine ratio, 1:2.8). Cells were transfected overnight in DMEM containing 10% (Melan-A Hm) or 5% FCS (PSM). On day 2, they were starved as described above and used on day 3 for the transwell assay. All transwell assays were done at least thrice independently, and migration rate was indicated as fold migration compared with untreated controls.

Results

To find out if Xmrk activation can induce migration, we first made use of Melan-A Hm cells in which the Xmrk activity can be induced by EGF. As we wanted to prevent the influence of Xmrk-unrelated events on migration, we used uncoated transwell inlays in migration assays. The migration rate of Melan-A Hm cells was determined in the range of 1 ng/mL to 10 μg/mL EGF (Fig. 1) and the concentration that resulted in highest migration rates (100 ng/mL) was used for further experiments. This effect was reliably dependent on HERmrk, because in the parental cell line Melan-A migration cannot be induced by EGF (data not shown).

Inhibition of the Xmrk kinase activity in the HERmrk chimera using AG555, a specific inhibitor of EGFR proteins including Xmrk (34), shows a reduction of EGF-induced migration to one third compared with the control and confirms that migration is
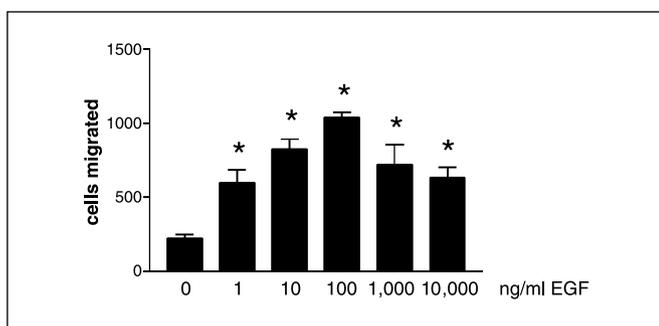
Figure 1. EGF induces melanocyte motility. Melan-A Hm cells were serum starved for 24 hours, and 2.5×10^4 cells were applied to the upper chamber of a 25-mm-diameter transwell inlay with 8 μm pores. EGF (0, 0.001, 0.01, 0.1, 1, or 10 μg/mL) was applied to the lower chamber. After 12 hours, the cells were stained with crystal violet and the number of migrated cells was counted. *Columns,* absolute number of migrated cells. *, $P < 0.05$.

dependent on the Xmrk kinase activity (Fig. 2*A*). As fyn and phosphatidylinositol 3-kinase (PI3K) interact with both FAK and Xmrk and reportedly play a role for cell motility (19, 37, 38), the effect of inhibiting these kinases was monitored in the migration assay. Although inhibition of PI3K had no effect on migration, src kinases seemed to be required for HERmrk-induced motility because the src kinase inhibitor PP2 reduced migration as efficient as AG555 (Fig. 2*A*). Fyn is the only member of the src family that is activated by Xmrk (39). To validate that fyn is involved in HERmrk-mediated migration, we expressed a dominant-negative fyn consisting of the fyn SH2 domain in Melan-A Hm cells. Fyn-SH2 reduced the migration to ∼55%, whereas the control vector had no effect (Fig. 2*B*). As the transfection efficiency was ∼50%, a reduction to background levels could not be expected.

To determine the relevance of FAK for HERmrk-mediated melanocyte motility, we transfected Melan-A Hm cells with a dominant-negative version of FAK (the FAK-related nonkinase FRNK) containing only the COOH-terminal part of FAK. It localizes to focal adhesions due to the presence of the FAT domain, but lacks the kinase domain and the NH_2-terminal part that interacts with growth factor receptors (40). The presence of FRNK reduced the EGF-induced migration of Melan-A Hm cells to 40% (Fig. 2*C*).

The src kinase fyn plays a prominent role for Xmrk signaling and transformation (31, 32, 36). As src kinases bind strongly to FAK via SH2 or SH3 domains, the involvement of fyn was analyzed. In fyn immunoprecipitates with subsequent anti-PY probing, phosphorylated HERmrk could be detected only when cells had been treated with EGF (Fig. 3*A*). Phospho-FAK also coprecipitated with fyn, but both binding of phospho-FAK and whole FAK did not change in response to EGF (Fig. 3*A*). The data show that FAK bound to fyn in an EGF-independent manner, but that there was a complex of active HERmrk, fyn, and FAK in EGF-stimulated cells.

To confirm the FAK HERmrk interaction seen in fyn precipitates, FAK immunoprecipitates of Melan-A Hm cells were analyzed for coprecipitation of the receptor. A 2-hour stimulation led to an increased binding of HERmrk to FAK, but also to a reduced amount of phosphorylated FAK (Fig. 3*B*). The regulatory domain of PI3K, p85, also binds to FAK via its SH2 domain (38, 41). Similar to fyn, p85 is an interaction partner for Xmrk (32, 42). A p85 immunoprecipitation from Melan-A Hm cells revealed almost no interaction with FAK (Fig. 1*D*). This outcome was independent of EGF stimulation but did not result from low amounts of either p85 or FAK in the cell line (Fig. 3*C*).

As a 2-hour incubation of Melan-A Hm cells resulted in dephosphorylation of FAK, the effect of different EGF concentrations and stimulation times on FAK phosphorylation was investigated. EGF (1, 10, or 100 ng/mL) was applied for 10 minutes or 2 hours. The phosphorylation of FAK was highly variable (Fig. 4*A*). After short stimulation with intermediate and high EGF concentrations (10 or 100 ng/mL), FAK became phosphorylated (*lanes 3 and 4*). After 2 hours of stimulation, a clear decrease of phosphorylated
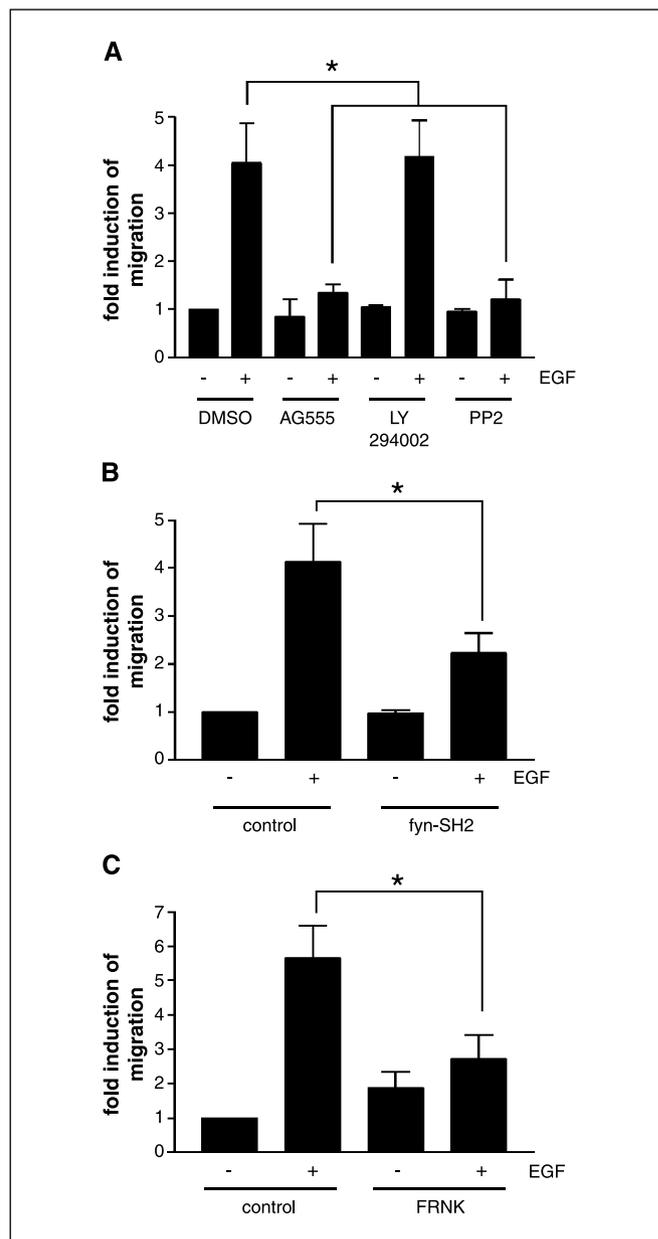
Figure 2. Inhibition of EGF-induced cell motility by FRNK and fyn. *A,* Melan-A Hm cells were serum starved for 24 hours and a transwell assay with 100 ng/mL EGF was done as described in Fig. 1. In addition, 20 μmol/L AG555, 20 μmol/L src inhibitor PP2, or 10 μmol/L PI3K inhibitor LY294002 were applied to the upper chamber in addition to the cells. *Columns,* fold migration of the inhibitor-treated samples compared with the untreated control. *B* and *C,* Melan-A Hm cells were transiently transfected either with pRK5 control vector or pRK5-GST-Xfyn2 (*B*) or pcDNA3 control vector or pcDNA3-FRNK (*C*). One day after transfection, the cells were serum starved for 24 hours and a transwell migration assay as described in Fig. 1 was done in the absence or presence of 100 ng/mL EGF. *, $P < 0.05$.
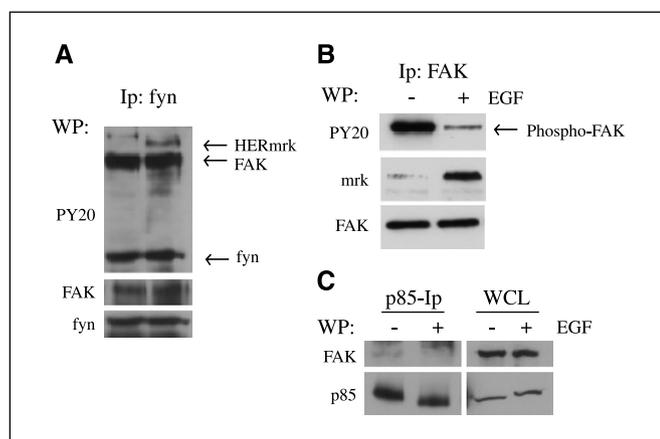
Figure 3. FAK interacts with HERmrk and fyn in response to EGF stimulation. Melan-A cells stably transfected with HERmrk were serum starved for 24 hours. Cells were treated with 100 ng/mL EGF for 2 hours. *A,* Melan-A Hm cells were incubated with EGF or left untreated. A fyn immunoprecipitation followed by a Western blot was done. The blot was first probed with PY20, then stripped and reprobed with FAK or fyn antibody (loading control). *B,* the cells were treated as described in (*A*), followed by a FAK immunoprecipitation and Western blot. The blot was first probed with PY20, then stripped and reprobed with mrk or FAK antibody (loading control). *C,* Melan-A Hm cells were incubated with EGF or left untreated, and a p85 immunoprecipitation was done as described above. The Western blot was probed against FAK and p85 (loading control). As a control for successful blotting and antibody recognition, 50 μg Melan-A Hm cell lysate of unstimulated or EGF-stimulated cells were loaded on the same gel as the immunoprecipitate. *Ip,* immunoprecipitation; *WP,* Western probe; *WCL,* whole cell lysate.

FAK was observed (*lanes 6* and *7*). Compared with that, the time-dependent change of the FAK phosphorylation state with low EGF concentrations was rather weak (*lanes 2* and *5*).

These data could indicate that activation of the receptor increased focal adhesion dynamics and turnover. To analyze this further, focal contacts in EGF-stimulated cells were monitored by FAK immunofluorescence. In untreated Melan-A Hm cells, FAK staining was very prominent in the cytosol, in close proximity to the nucleus, but also in peripherally located distinct spots that constitute the focal contacts (Fig. 4*B, arrows*). After 10 minutes of EGF treatment (100 ng/mL), FAK distribution and focal adhesions of the cells had a similar appearance compared with the untreated controls (Fig. 4*B, arrows*). When the cells were EGF-treated for 2 hours, the number of focal adhesions was remarkably reduced and FAK signal was almost exclusively found in the cytosol, with almost no distinct focal adhesions visible (Fig. 4*B*). In addition, 35% to 50% of the cells obtained a spindle-like appearance. An analysis of the cell shape reveals that long-term EGF stimulation resulted in a 14.4% decrease of relative attached cell surface (Fig. 4*C*).

The controls and the short-term stimulated cells were tightly attached to the cell culture dish, which was apparent as a continuous ring of focal adhesions around the cells. In long-term–treated cells, this ring was rarely visible, indicating focal adhesion disassembly. When cells were incubated with AG555 in addition to EGF, the number of FAK-positive focal contacts was comparable with the control (Fig. 4*B*). A similar effect was observed with PP2-inhibited cells, but to a lesser extent (Fig. 4*B*).

As focal contact turnover should be paralleled by major changes in the cytoskeleton, the stress fiber content was examined by phalloidin staining. Compared with the untreated controls, the stress fiber content of long-term stimulated Melan-A HERmrk cells was reduced (Fig. 5). The presence of AG555 in the EGF-stimulated

cell culture prevented this reduction and the stress fibers were maintained.

In summary, we showed that activation of the Xmrk kinase activity in HERmrk-expressing murine Melan-A cells resulted in the formation of a receptor/Fyn/FAK complex, activation of FAK, and increased focal contact and actin cytoskeleton turnover.

To investigate further whether the Xmrk receptor regulates migration in a similar manner in *Xiphophorus* melanoma, we used PSM cells, a *Xiphophorus* melanoma cell line that strongly over-expresses Xmrk. First, we did a migration assay as described above. As Xmrk is permanently active in PSM cells and does not respond to EGF, we used instead 10% FCS as a "chemoattractant" to induce a directed movement of the cells into the lower migration chamber. Cell migration was done either in the presence of DMSO or the inhibitors AG555, LY294002, or PP2, which are active in *Xiphophorus* cells as described previously (34, 42). As the PSM cells are kept on 28°C, the migration assay was stopped after 20 hours instead of 12 hours. PSM cell migration was inhibited by AG555, which shows that it is dependent on Xmrk (Fig. 6*A*). Similar to the situation in Melan-A cells, the inhibition of PI3K by LY204002 did not have any effect on migration, whereas PP2 blocked it completely.

To assess the importance of both FAK and fyn for PSM migration, we transfected the dominant-negative FAK and fyn constructs and did another migration assay. Both the fyn-SH2 and the FRNK domains are very well conserved between the species, which makes the constructs applicable both for the transfection in mouse and fish cells. The visible transfection efficiency estimated by counting enhanced green fluorescent protein–expressing cells under the microscope was 35%. Inhibition with FRNK and fyn-SH2 decreased migration to 62% and 45%, respectively, and confirmed the results observed with Melan-A Hm cells (Fig. 6*A*).

The protein-protein interaction between Xmrk and FAK could also be confirmed in PSM cells. Analysis of FAK immunoprecipitates with a Xmrk-specific antibody and with antiphosphotyrosine revealed that Xmrk is the most abundant phosphoprotein interacting with FAK (Fig. 6*B*).

As no ligand-independent activation of Xmrk was possible, we did an anti-FAK immunofluorescence with untreated and AG555-treated PSM cells to reduce Xmrk activity (34). The appearance of focal contacts in the fish cells is different from Xmrk-transformed mouse melanocytes. Focal contacts cannot be visualized all over the cell body, but only at cell protrusions (Fig. 6*C, arrows*). After inhibition of Xmrk with AG555, these focal contacts became clearly larger and more distinct (Fig. 6*C, arrows*).

FITC-phalloidin staining revealed that the filamentous actin (F-actin) framework is very weak in this cell type, which made it hard to see a difference between untreated and AG555-treated PSM cells (data not shown). We therefore induced the cells with FCS to strengthen the stimulus for cell movement and possibly allow a better distinction in terms of stress fiber formation. Interestingly, this treatment led to the appearance of numerous filopodia that disappeared in presence of AG555 (Fig. 6*D, arrows*). In addition, the amount of stress fibers was increased by the inhibitor, but the fibers were still weaker than the ones observed in Melan-A Hm cells.

Discussion

FAK is responsible for motile and invasive cell behavior and its activity is up-regulated in a number of tumors (43–46). Here, we show that the oncogenic EGFR variant Xmrk, which is responsible for the development of highly malignant tumors in *Xiphophorus* fish,

modulates the activity of FAK and that this modulation is important for melanocyte motility. The interaction between Xmrk and FAK only occurs after receptor activation. This suggests that phosphorylation of the receptor is either directly involved in this process or occurs via mediator proteins like fyn. It is known that the NH$_2$-terminal FERM domain of FAK is important for signal integration from growth factor receptors, such as EGFR, platelet-derived growth factor receptor, Ephrin kinases, and the cytoplasmic epithelial and endothelial tyrosine kinase, and might directly interact with the catalytic domain of FAK (12). However, our data indicate that fyn, but not p85, can also play a role for receptor-mediated FAK modulation. The importance of fyn as a malignancy marker has already been described in mouse melanoma (24). In our system, fyn binds both active HERmrk and FAK after EGF stimulation and might, therefore, bring these molecules in close proximity to each other or strengthen their preexisting interaction. Either this close proximity of HERmrk and adjoining proteins or factors influenced by HERmrk-activated fyn are affecting FAK activity.

As the immunoprecipitation results show, there is no direct influence of fyn on FAK phosphorylation. In agreement with these data, experiments with kinase-deficient src in colon carcinoma cells show that FAK phosphorylation occurs independently of src kinase activity (14). The authors even report an increased FAK phosphorylation after expression of a kinase-deficient src variant. The phosphorylations occurred at tyrosine residues that are considered typical src kinase substrates. These data support the idea that src can act as an intermediate protein that recruits phosphorylating or dephosphorylating proteins to FAK and are in accordance with our results for the related src kinase fyn. In addition, data from reconstituted FAK−/− cells reveal that src recruitment to FAK is an initial event leading to focal contact turnover and enhanced cell motility (40, 47). Our immunofluorescence studies show that both the inhibition of HERmrk and of fyn lead to the stabilization of focal contacts and prevent their turnover, confirming a similar role for fyn.

Although fyn itself does not change FAK phosphorylation in our assays, the kinase activity of fyn is clearly required for cell motility.
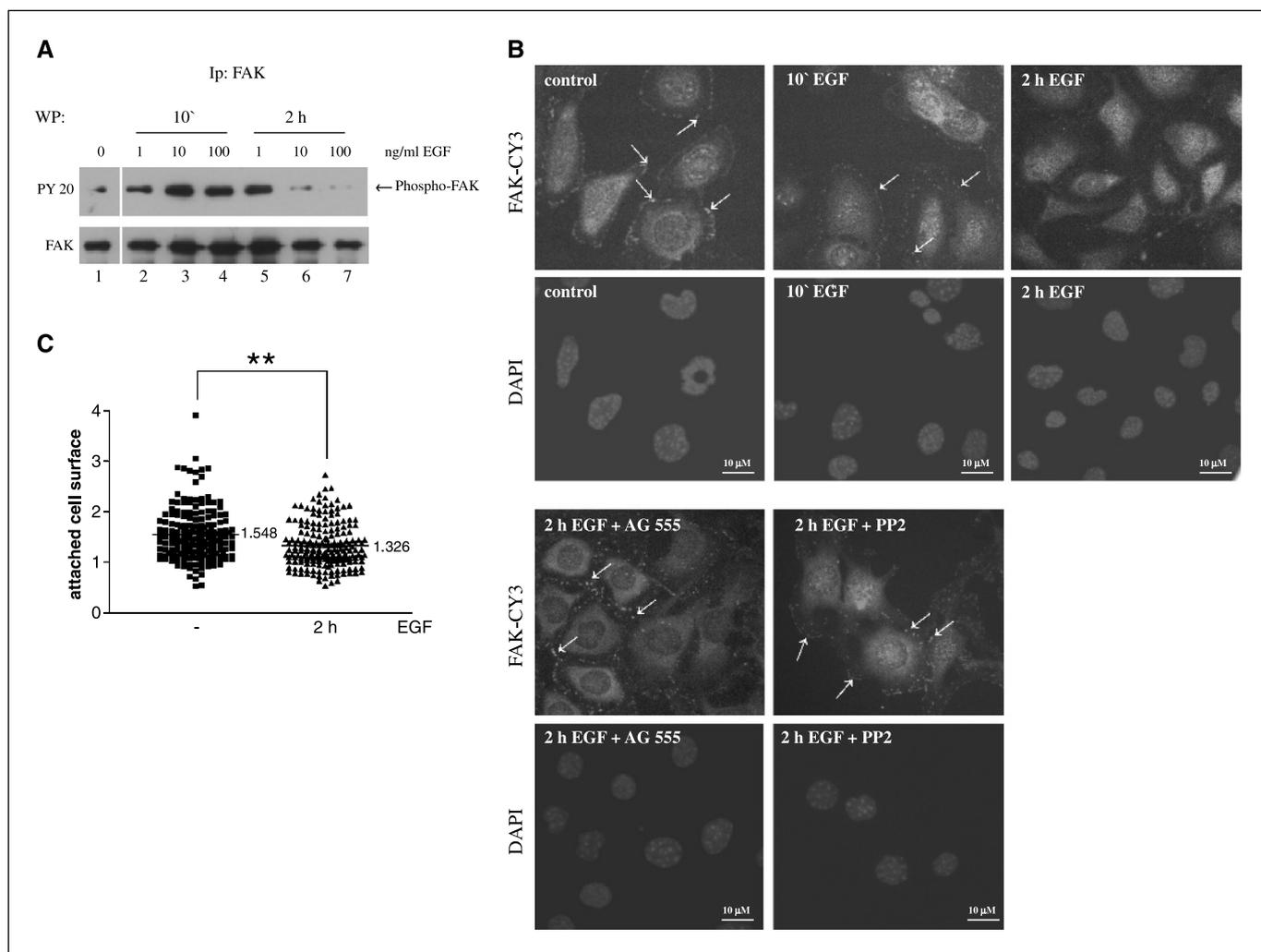
Figure 4. Activation of Xmrk-modulated FAK phosphorylation and focal adhesion turnover. *A,* Melan-A Hm cells were serum starved for 24 hours and incubated with 1, 10, or 100 ng/mL EGF for 10 minutes or 2 hours. They were used for FAK immunoprecipitation, SDS-PAGE, and Western blot analysis as described in Fig. 3. *B,* Melan-A Hm cells were grown on glass coverslips and serum starved for 24 hours. They were either left untreated, incubated with 100 ng/mL EGF for 10 minutes or 2 hours, or incubated for 1 hour with 20 μmol/L of the EGFR inhibitor AG555 or the 20 μmol/L of the src kinase inhibitor PP2 before the 2-hour EGF stimulation. The cells were fixed, permeabilized, and an immunofluorescence staining was done with anti-FAK as first antibody and anti-rabbit-CY3 as second antibody. *C,* for a comparison of the attached cell surface, Melan-A Hm cells in unstimulated state or after 2 hours of EGF addition (100 ng/mL) were photographed and analyzed with help of the NIH image software. **, $P < 0.01$.

This becomes apparent by the strongly reduced migration after addition of the inhibitor PP2. As neither src nor yes is activated by Xmrk (39), it is unlikely that the blocking effect of PP2 on migration is mediated by an inhibition of these kinases. In addition, migration assays with the SH2 domain of fyn, acting as a dominant-negative fyn version, confirmed the importance of this protein for cell motility. A similar observation was made for metastatic murine melanoma (24). In this work, fyn was the only member of the src kinases that was activated in highly metastatic, but not in lowly metastatic, cells and led to the activation of the cytoskeleton-linker protein cortactin.

Although an increased FAK activity has been observed in many malignant melanoma and seems to be crucial for metastasis, no FAK mutations that lead to constitutive activation and only moderately increased expression levels have been observed (8, 43–46). Obviously, the regulation of FAK is a result of upstream molecules like integrins or receptor tyrosine kinases. In adhesive cells, the focal contacts confer substrate adhesion and a high number of focal adhesions result in a strong attachment to the substratum. In migrating cells, focal contacts serve as sites for force transmission against the substratum, and actin-myosin–dependent processes transfer the force through the cell body. Therefore, the reduction of focal adhesions is advantageous for a tumor cell that detaches from its natural surrounding, whereas concerted attachment/detachment processes and consecutive focal adhesion turnover are crucial for migration. This requires a fine-tuned regulation of focal contact proteins, which is not yet fully understood. We could show that the EGF-dependent reduction of focal adhesions is accompanied by changes in cell shape that result in a reduced surface attaching the substratum, thereby possibly facilitating cell movement.

Among the RTKs that have been found to regulate FAK are insulin-like growth factor-I and EGFR. It has been reported that low or intermediate concentrations of EGF (1-10 ng/mL), applied for short time periods, lead to a phosphorylation of FAK, whereas high concentrations tend to induce dephosphorylation (11, 48). In addition, a time-dependent change in FAK phosphorylation after application of 10 ng EGF/mL has been observed in adenocarcinoma cells (49). Ojaniemi and Luori (50) reported for Cas another focal adhesion protein downstream of FAK, a bell-shaped dose-response curve by EGF, with phosphorylation at lower dosages and dephosphorylation at higher concentrations. The different outcomes for the cell lines may be the result of different receptor densities or cell-specific variations in receptor internalization, leading to different degrees of feedback regulation. In our cell system, FAK phosphorylation was dose- and time-dependent, too. For EGFR-induced motility, two phases of focal adhesion integrity have been observed. In EGFR-overexpressing carcinoma cells, an EGFR-dependent FAK down-regulation is required for morphologic changes and detachment from the extracellular matrix, but FAK activity is restored upon reattaching by activated integrin signaling (11). The regulation of focal adhesion turnover is, therefore, complex and can be influenced by different stimuli.

When we reduced the amount of integrin-localized FAK by transient overexpression of FRNK, the EGF-induced cell motility in Melan-A Hm cells was highly impaired. Unexpectedly, the presence of FRNK increased the background levels of motility This might be explained by the prevention of a proper assembly of focal adhesion proteins, thereby reducing the number of focal contacts on the control cells. Immunofluorescence of FRNK-transfected Melan-A Hm cells revealed that the number of focal contacts was indeed reduced in FRNK-transfected cells (data not shown). As a result, the attachment to the transwell membrane was reduced, which might have helped the cells to pass through the pores. For EGF-induced motility, both deactivation and activation processes at the focal contacts are important for regulating cell traction (11). Therefore, the motility was higher compared with control cells transfected with FRNK. Thus, the reactivation of FAK during the migration process appears also regulated by Xmrk and is required for motility.

In contrast to the EGF-inducible HERmrk model system, Xmrk is constitutively active at the site of its natural occurrence, the *Xiphophorus* melanoma. PSM cells isolated from *Xiphophorus* melanoma depend on the same migratory pathways as Melan-A Hm cells, as inhibition experiments and the transfection of dominant-negative fyn and FAK constructs revealed. Immunofluoresence analysis showed that Xmrk-overexpressing PSM cells possess much weaker focal contacts compared with the kinase-inhibited control. This suggests that a high focal contact turnover is also found in cells with permanently active Xmrk. This assumption is supported by the visualization of F-actin by phalloidin-FITC. The stress fibers are very weak in PSM cells, but they become stronger after the specific inhibition of Xmrk by AG555. Surprisingly, the Xmrk-dependent lack of visible F-actin inside the cell is contrasted by its appearance in filopodia-like structures at the cell periphery after serum stimulation. This leads to the conclusion that Xmrk is also involved in major cytoskeletal
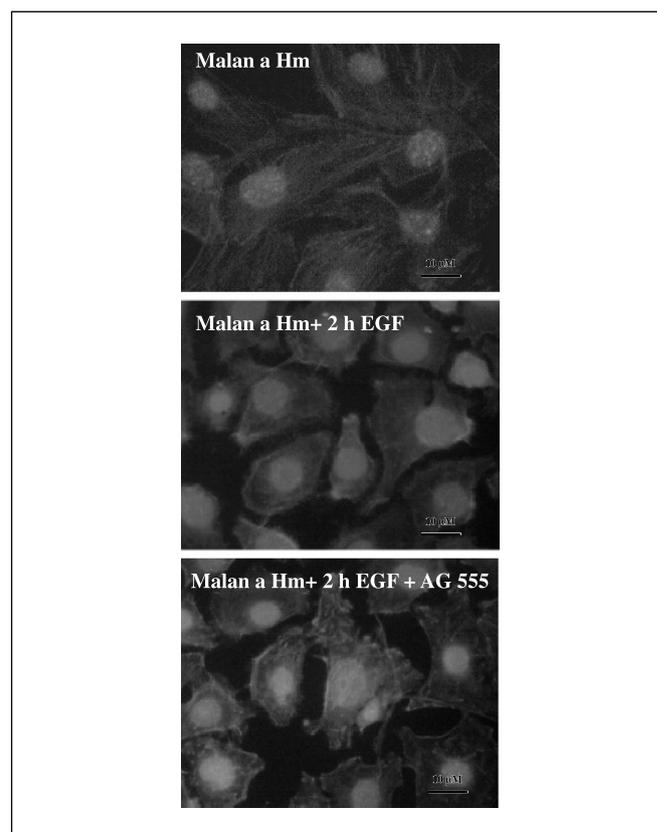
Figure 5. Reduction of actin stress fibers in melanocytes after EGF treatment. Melan-A Hm cells (2×10^5) were grown on glass coverslips and serum starved for 24 hours. The slides were either left untreated or incubated with EGF for 2 hours with 100 ng/mL either in the absence or presence of 20 μmol/L AG555 (applied 1 hour before the growth factor). Samples were fixed, permeabilized, and stained with FITC-conjugated phalloidin that recognizes F-actin.
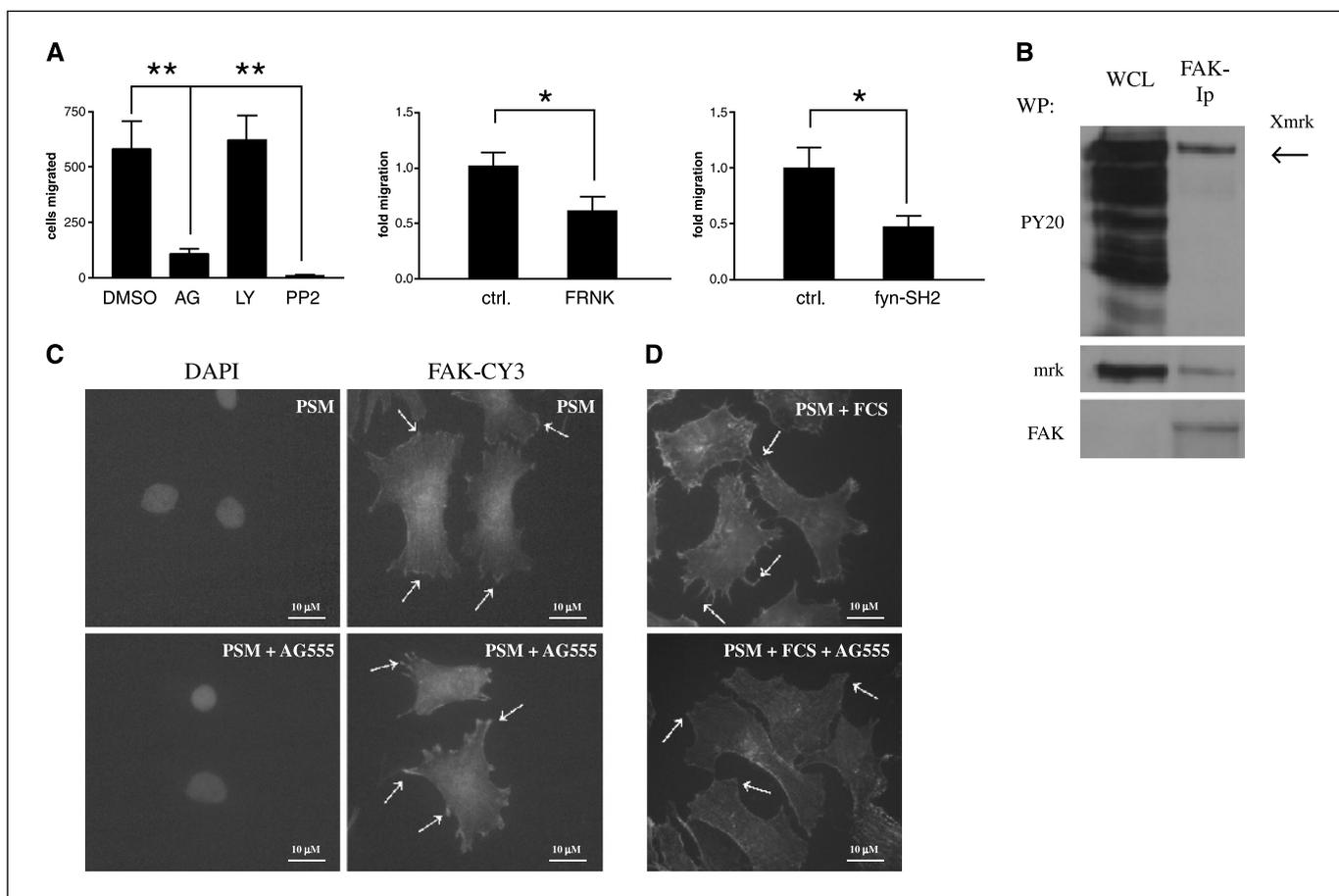
Figure 6. Migration and detection of focal contacts in the *Xiphophorus* melanoma cell line PSM. *A,* PSM cells were serum starved for 24 hours, and 2.5×10^4 cells in presence of either DMSO or the indicated inhibitors were applied to the upper chamber of a 25-mm-diameter transwell inlay with 8 μm pores. FCS (10%) was applied to the lower chamber. After 20 hours, the cells were stained with crystal violet and the number of migrated cells was counted. *Columns* (left), absolute number of migrated cells. In addition, PSM cells were transfected with either pRK5, pRK5-GST-Xfyn2, pcDNA3, or pcDNA3-FRNK in addition to pEGFP as a transfection control. Twenty-four hours after transfection, the cells were serum starved for 24 hours, and a transwell assay was done as described above. *Columns* (*middle and right*), fold migration of cells compared with the vector controls. *, $P < 0.05$; **, $P < 0.01$. *B,* anti-FAK immunoprecipitates or whole cell lysate of PSM cells were blotted onto nitrocellulose and subsequently probed with the antiphosphotyrosine antibody PY20. The blot was stripped and then reprobed with antibodies against Xmrk and FAK, the latter as a control for the success of the FAK-specific immunoprecipitation. *C,* 2×10^5 PSM cells were grown on glass coverslips and serum starved for 24 hours. They were either treated with DMSO or with 20 μmol/L AG555 for 3 hours before fixation and subsequent anti-FAK immunofluorescence. *D,* phalloidin-FITC staining of PSM cells. The cells were serum starved as described above and treated for 3 hours with DMSO or 20 μmol/L AG555. During the last 2 hours, the cells were additionally treated with 10% FCS.

rearrangements independent of the disassembly of focal contacts and the associated stress fibers.

Together, our data show that Xmrk induces FAK-dependent motility in pigment cells from mouse and fish origin, which is mediated by activated fyn, and that successive activation and deactivation processes are crucial for this event. A schematic overview of the migration-relevant pathways in Melan-A Hm and PSM cells is presented in Supplementary Fig. S1.

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The Oncogenic Epidermal Growth Factor Receptor Variant *Xiphophorus* Melanoma Receptor Kinase Induces Motility in Melanocytes by Modulation of Focal Adhesions

Svenja Meierjohann, Elisabeth Wende, Anita Kraiss, et al.

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