The conformational state of hERG1 channels determines integrin association, downstream signaling, and cancer progression

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ABSTRACT

Ion channels regulate cell proliferation, differentiation, and migration in normal and neoplastic cells through cell-cell and cell-extracellular matrix (ECM) transmembrane receptors called integrins. K^+ flux through the human ether-à-go-go-related gene 1 (hERG1) channel shapes action potential firing in excitable cells such as cardiac myocytes. Its abundance is often aberrantly high in tumors, where it modulates integrin-mediated signaling. We found that hERG1 interacted with the β1 integrin subunit at the plasma membrane of human cancer cells. This interaction was not detected in cardiac myocytes because of the presence of the hERG1 auxiliary subunit KCNE1 (potassium voltage-gated channel subfamily E regulatory subunit 1), which, blocked the β1 integrin-hERG1 interaction. Although open hERG1 channels did not interact as strongly with β1 integrins as did closed channels, current flow through hERG1 channels was necessary to activate the integrin-dependent phosphorylation of Tyr^{397} in Focal Adhesion Kinase (FAK) in both normal and cancer cells. In immunodeficient mice, proliferation was inhibited in breast cancer cells expressing forms of hERG1 with impaired K^+ flow whereas metastasis of breast cancer cells was reduced when the hERG1/β1 integrin interaction was disrupted. We conclude that the interaction of β1 integrins with hERG1 channels in cancer cells stimulated distinct signaling pathways that depended on the conformational state of hERG1 and affected different aspects of tumor progression.

INTRODUCTION

Cell adhesion to the extracellular matrix (ECM) is critical to various processes, including cell migration, synaptic plasticity, and the decision between proliferation and differentiation (*1, 2*). Both voltage- and ligand-gated ion channels regulate how cells respond to the ECM by interacting

with integrin receptors (*3, 4*). Integrin receptors are transmembrane proteins consisting of various α and β subunits. In mammals, they can form more than twenty different heterodimers with specific ECM binding patterns (*5, 6*). Under resting conditions, integrin receptors reside in a folded low-affinity conformational state. Upon activation by extracellular or intracellular ligands or binding partners, they shift to a high-affinity extended conformation (*7, 8*), which mediates both 'outside-in' and 'inside-out' signaling (*5*). An important early signal triggered by integrin engagement is phosphorylation of focal adhesion kinase (FAK), which in turn regulates downstream signaling (*9*).

A common molecular partner of integrin receptors is the human ether-à-go-go-related gene 1 protein (hERG1, also known as $K_v11.1$). In cardiac myocytes, hERG1 mediates the cardiac repolarizing current I_{kr} (*10*) in association with accessory subunits such as the potassium voltage-gated channel subfamily E regulatory subunit 1 (KCNE1, also known as MinK1). Moreover, hERG1 regulates excitability in the central nervous system, endocrine cells and smooth muscle (*11, 12*). However, information about the interaction between integrin receptors and hERG1 is available only for neoplastic cells. The abundance of hERG1 is often aberrantly high in human cancers, and this channel is implicated in different stages of neoplastic progression, such as cell proliferation and survival, invasiveness, and neo-angiogenesis (*12-14*). Because these processes are regulated by cell adhesion to ECM, the crosstalk between integrin receptors and hERG1 may constitute a unifying modulatory mechanism of the cellular response to the microenvironment in both normal and tumor tissue (*4, 15*).

Integrin receptors and ion channels communicate by diffusible signals (*16-20*) as well as by forming macromolecular complexes (*21-25*). In particular, the β1 integrin-mediated adhesion to fibronectin activates hERG1 currents (I_{hERG1}) in different cell types (*17, 26*). In neuroblastoma cells, this process is mediated by $G\alpha_i$ protein (*17*) and triggers integrin-dependent signaling cascades (*23, 26*). In addition, cell adhesion to fibronectin generally stimulates the formation of a macromolecular complex between hERG1 and β1 integrin (hERG1/β1 integrin complex) (*23*), which may recruit growth factor receptors such as the vascular endothelial growth factor receptor-1 (VEGFR-1) (*27*),

and the C-X-C motif chemokine receptor 4 (CXCR-4) (*28*). Therefore, hERG1 takes part in multi-protein complexes that constitute major signaling centers in different cell types.

How hERG1 and β1 integrin interact and how the ensuing macromolecular complex responds to diffusible signals is unknown. The role of K^+ flow as compared to non-conductive hERG1-related signals is also unclear. Here, we first investigated why the hERG1/β1 integrin complex was formed in cancer cells, but not in heart tissue, where hERG1 is found with its ancillary subunits (*11*). Next, we studied the mechanism of interaction of the two proteins using deletion mutants. The protein-protein interaction was further confirmed with Förster Resonance Energy Transfer (FRET) analysis (*29-32*). Using hERG1 mutants with alterations in either conductance or gating, we sought to discriminate between the contributions of the channel's conformational state and K^+ flow to the assembly of the macromolecular complex and to downstream signaling. Finally, we studied the in vivo effects of disrupting the interaction between hERG1 and β1 integrin on the growth and metastasis of breast cancer cells xenografted in mice.

RESULTS

KCNE1 and β1 integrin compete for binding to hERG1

We compared the interaction between hERG1 and β1 integrin in primary cancer samples and surgical samples from human hearts. β1 integrin immunoprecipitates from colorectal cancers (CRC), pancreatic cancers (PC), acute myeloid (AML) and chronic myeloid (CML) leukemias contained a hERG1 band (**Fig. 1A**, left panel). Conversely, a β1 integrin band was detected in hERG1 immunoprecipitates from CRC, PC and CML-1 samples (**Fig. 1A,** right panel). In contrast, no association between hERG1 and β1 integrin was detected in cardiac tissue from human atria (**Fig. 1B**). Because hERG1 associates with KCNE1 in cardiac myocytes (*10, 33*), we hypothesized that the ancillary protein could impair the channel interaction with β1 integrin. We first verified that both hERG1 (**Fig. 1B**, input hERG1) and KCNE1 (**Fig. 1C**) were present in the heart samples. In contrast, neither the *KCNE1* transcript nor the corresponding protein was found in the tumor cell lines with a hERG1/β1 integrin complex (**Fig. 1C**) (*26, 27*). Next, we expressed KCNE1 in HCT116 cells, which have endogenous hERG1, or HEK 293 (HEK) cells, which lack the potassium channel and were transfected with hERG1. The presence of transcript for KCNE1 was verified by Real Time Quantitative PCR (RT-qPCR) (**Fig. S1, A and B**). In HEK cells plated on fibronectin, the formation of the hERG1/β1 integrin complex was inhibited by KCNE1 (**Fig. 1D**). Similar results were obtained in HCT116 cells (**Fig. 1E**), although the effect was smaller. These results suggested that KCNE1 and β1 integrin competed for binding to hERG1, which could explain why formation of the hERG1/β1 integrin complex occurs only in neoplastic tissue.

hERG1 and β1 integrin may directly physically interact

To study how hERG1 and β1 integrin assemble, we used HEK cells transfected with wild-type (HEK-hERG1) or mutant hERG1 cDNA. Endogenous β1 integrin is present in these cells and co-immunoprecipitated with hERG1 in cells allowed to adhere to fibronectin (*23*) (**Fig. S2**). To study

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whether the two proteins interacted directly, we applied Hyperspectral-Fluorescence Lifetime Imaging Microscopy-FRET (HS-FLIM-FRET) (*34*), on HEK cells transfected with CFP-labeled hERG1 and YFP-labeled β1 integrin (**Fig. S3)**. The fluorescence decay was characterized by bi-exponential behavior with one long-lived and one fast decaying component. From each cell (**Fig. 2A**), we obtained the lifetimes (τ_s and τ_f, respectively) and fraction size (A_s and A_f) of both components for all pixels of the image (control experiments are in **Fig. S4** and **Fig. S5**). The slow lifetime was similar to the lifetime of eCFP when no energy transfer occurred ($\tau_s = 2.48 \pm 0.09$ ns, see Supplementary Materials). The fast lifetime is a signature of energy transfer and the close proximity of hERG1 to β1 integrin. By using equation (2) (Supplementary Materials and Methods), the calculated FRET efficiency was 0.77 ± 0.04. We also compared the HS-FLIM-FRET signals in cells cultured onto fibronectin or bovine serum albumin (BSA). Measurements were stopped at 100 min, because after that time cells cultured onto BSA progressively detached from the substrate. The donor mean lifetime values (τ_m) was calculated using eq. (3) (Supplementary Materials). For cells cultured on fibronectin τ_m was 1.71 ± 0.05, whereas for cells cultured on BSA τ_m was 2.00 ± 0.08 (**Fig. 2B**). Shorter lifetimes in cells cultured onto fibronectin indicated higher FRET efficiency in elongated cells that strongly adhered to the substrate (**Fig. 2C**, FRET). In contrast, longer lifetimes, which indicated lower FRET efficiency or no FRET, corresponded to round cells or cells fixed to the substrate through small filopodia (**Fig. 2C**, NO FRET).

The hERG1/β1 integrin complex is localized to the plasma membrane

To demonstrate that hERG1 and β1 integrin interaction occurred at the plasma membrane, the functional site of both proteins, we performed FRET experiments by acceptor photobleaching imaging (*29*) on fixed HEK cells. Donor and acceptor were labeled using monoclonal antibodies directed against hERG1, which was coupled to AlexaFluor488, and against β1 integrin, which was coupled to AlexaFluor546 (**Fig. S6** and **Fig. S7**). Confocal imaging allowed us to image FRET at various locations in the cell. We chose two different focal planes, one located at the plasma

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membrane close to the fibronectin-coated substrate (**Fig. 2D, Bottom plasma membrane**), and one located at approximately half of the maximal cell thickness (**Fig. 2D, Z-slice plasma membrane**). Data analysis confirmed that the FRET signal originated on, or was proximal to, the plasma membrane (acceptor mask in **Fig. S7B**). The average FRET efficiencies calculated for the bottom and the equatorial (Z-slice) plasma membranes were similar (**Fig. 2E**). Hence, the interaction between hERG1 and β1 integrin was not restricted to the membrane portions involved in cell-substrate adhesion, but also occurred in membrane patches distant from the adhesion sites. The mean FRET efficiency for both locations was 0.24 ± 0.05, about 4-fold smaller than the value measured by HS-FLIM-FRET, which we attribute to the different labeling methods for hERG1 and β1 integrin.

Interaction between hERG1 and β1 integrin does not require the hERG1 intracellular domains or the cytoplasmic C-terminal domain of β1 integrin

To define the molecular domains implicated in complex formation, we performed electrophysiological analysis of HEK cells stably expressing forms of hERG1 lacking the entire N-terminus (hERG1Δ2-370) or the C-terminus (hERGΔC+RD) except for the amino acids from 1018 to 1122, which constitute the Recapitulation Domain (RD) that allows hERG1 insertion into the plasma membrane (*35*). As expected, whole-cell current recordings indicated that hERG1Δ2-370 displayed the typical fast deactivation conferred by N-terminus deletion (*36*), whereas hERG1ΔC+RD generally displayed current amplitudes considerably smaller than those of hERG1 (*35*) (**Fig. 3A**). Immunoprecipitation analysis of these cells suggested that the cytoplasmic hERG1 domains were dispensable for assembly with β1 integrin (**Fig. 3B**). To test if the intracellular domain of β1 integrin interacted with hERG1, we transfected hERG1 and the following YFP-labeled constructs into GD25 cells, which are deficient in β1 integrins (*37*): (i) full-length β1 integrin, (ii) β1 integrin lacking the C-terminus (β1-extra) and (iii) β1 integrin C-terminus linked to

the transmembrane and extracellular portions of the interleukin-2 receptor (β1-cyto) (**Fig. 3C, 3D**).

Co-immunoprecipitation analysis showed that hERG1 co-immunoprecipitated with β1 integrin and β1-extra, but not with β1-cyto (**Fig. 3D**), suggesting that the cytoplasmic domain of β1 integrin was not necessary for interaction with hERG1. We confirmed this conclusion by performing co-immunoprecipitation using an antibody against β1 integrin in GD25-β1-TR cells, which stably express β1 integrin lacking the C-terminus (**Fig. 3E**). We concluded that the cytoplasmic domains of hERG1 and β1 integrin were not necessary for the interaction of these proteins.

HERG1 gating, but not conduction, regulates the complex formation.

The results obtained with hERG1ΔC+RD (**Fig. 3A and 3B**) suggested that impairing channel conduction did not affect the association of hERG1 with β1 integrin. To further investigate this issue, we tested the complex formation in the presence of E4031, which blocks hERG1 (**Fig. 4A)** by binding to the Phe^{656} residue that protrudes into the channel pore (*38*). E4031 inhibited the complex formation by about 80% (**Fig. 4B**). This result could be explained if K^+ flux is critical for complex formation, or if the presence of E4031 locks a substantial fraction of the channels in the open conformation, which may have a lower affinity for β1 integrin. To better distinguish these mechanisms, we expressed several mutant constructs in HEK cells: the non conducting hERG1-G628S (*38*), hERG1-R531C and hERG1-K525C, which are S4 domain mutants with altered activation (*39*), and the non-inactivating hERG1-S620T (*40*). Flow cytometry analysis (*41*) indicated that the plasma membrane abundance of the mutants was ~25-30% less compared to wild-type hERG1 (**Fig. 4C**). The strong decrease in K^+ flow for the hERG1-G628S mutant is not accompanied by gating alterations (*38*). hERG1-G628S current amplitudes were comparable to those displayed by hERG1 in the presence of E4031 (**Fig. 4D**). Cells expressing hERG1-G628S had a mean membrane voltage (V_m) in resting conditions (V_{rest}) of -36.9 ± 1.9 mV, compared to -48.4 ± 2.3 mV in cells expressing wild-type hERG1. Formation of the hERG1/β1 integrin complex was not

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significantly impaired by hERG1-G628S (**Fig. 4E**). Thus, although blocking channel conduction with E4031 impaired the complex assembly, data obtained with the non-conducting hERG1-G628S mutant suggested that decreased current flow per se was not the main determinant of the effect, in agreement with the results obtained with hERG1ΔC+RD.

To test the alternative hypothesis that the complex formation is sensitive to the channel's conformational state, we used mutants with different steady-state activation properties (**Fig. 5A**). Like wild-type hERG1, the hERG1-K525C protein was detected at the plasma membrane (**Fig. 4C**) although the maximal current density was lower (**Fig. 5B**) and the activation curve was shifted to more negative V_m (**Fig. 5C**). In agreement with previous work (*39*), the estimated $V_{1/2}$ of activation was around -50 mV, and the measured V_{rest} was -59.1 ± 1.1. Hence, at steady state, hERG1-K525C channels spend more time in the open state than wild-type hERG1. hERG1-R531C had a similar maximal current density to that displayed by wild-type hERG1 (**Fig. 5B**). The estimated $V_{1/2}$ of activation was approximately +35 mV (**Fig. 5C**). The corresponding V_{rest} was -42 ± 2.6 mV, implying that a large fraction of hERG1-R531C channels resided in the closed state in our cells. Co-immunoprecipitation analysis showed that less β1 integrin associated with hERG1-K525C than with wild-type hERG1 or hERG1-R531C (**Fig. 5D**). This result suggested that increasing the probability of the channel being in the open state decreased complex formation. To further test this hypothesis, we used the non-inactivating hERG1-S620T mutant, in which the open-closed transition is not complicated by the presence of the inactive state (*40*). Consistent with the absence of inactivation (**Fig.5A**), hERG1-S620T had a maximal current density of 447 ± 89 pA/pF (**Fig.5B**), considerably higher than that measured for wild-type hERG1, and the measured V_{rest} was -59 ± 2.3 mV. Similarly to hERG1-K525C, the complex association of hERG1-S620T with β1 integrin was impaired compared to wild-type hERG1 **(Fig. 5E).** These results are consistent with the hypothesis that assembly of the hERG1/β1 integrin complex was hindered when the probability of the channel transition towards the open state increased.

This mechanism was further investigated by FRET experiments in acceptor photobleaching setting, on HEK cells expressing either wild-type hERG1, or hERG1-R531C, or hERG1-K525C. The FRET efficiency distributions for the three conditions were not statistically different (**Fig. 5F**), demonstrating that energy transfer took place in both hERG1-R531C and hERG1-K252C transfected cells. This finding suggested that the hERG1/β1 integrin complex, once it formed, was structurally similar in wild-type and mutant channels. Next, we determined the percentage of FRET events, which reflected the abundance of hERG1/β1 integrin complexes. In agreement with the co-immunoprecipitation data, complex formation was significantly impaired in cells expressing hERG1-K525C (**Fig. 5G**). Remarkably, these findings did not depend on the relative ratio between donor (hERG1) and acceptor (β1 integrin) present in the membrane (**Fig. S8**).

hERG1 current flow regulates FAK phosphorylation and cell proliferation in vivo

We have previously shown that the β1 integrin-dependent FAK phosphorylation in HEK cells expressing wild-type hERG1 relies on hERG1 activation (*23*). Hence, we studied how this process was modified by either E4031 or by the mutant channels. FAK was immunoprecipitated from HEK cells expressing wild-type hERG1 (treated or not with E4031) or one of the mutant hERG1 channels (**Fig. 6A**). The phosphorylation of Tyr^{397} in FAK (*23*) was impaired in cells expressing mutant hERG1 channels, with the exception of hERG1-S620T, compared to that in HEK cells expressing wild-type hERG1 (**Fig. 6A**). This finding suggests that the integrin-dependent FAK auto-phosphorylation required physiological hERG1 current amplitudes, which was impaired for different reasons by hERG1-G628S, E4031, hERG1-K525C and hERG1-R531C, but not by hERG1-S620T. Because FAK controls various cellular functions, including cell survival and proliferation (*42*), by activating numerous signaling pathways, we tested the effects of the above treatments on in vivo cell growth. We subcutaneously injected HEK cells expressing wild-type hERG1, hERG1-G628S, hERG1-K525C or hERG1-R531C into immunodeficient nude mice. In mice that received cells expressing the different mutants or expressing wild-type hERG1 in the

presence of E4031, the growth of subcutaneous masses was reduced compared to mice injected with HEK cells expressing wild-type hERG1 (**Fig. 6B**).

Disrupting the hERG1/β1 integrin complex inhibits metastasis of breast cancer cells

We next tested whether such hERG1-mediated cell signals were present in cancer cells in which the abundance of hERG1 is abnormally high and in which hERG1 forms a macromolecular complex with β1 integrin (*26, 28*). Specific hERG1 blockade with either Way 123,398 (WAY) or E4031 impairs the phosphorylation of Tyr^{397} in FAK (*23,26-28*). This process was also studied in MDA-MB-231 breast cancer cells. Because these cells have low amounts of hERG1, we transfected our wild-type and mutant hERG1 constructs, which approximately doubled the membrane amount of hERG1 channels (**Fig. 7A**). Once again, only hERG1-K525C strongly impaired the hERG1/β1 integrin complex formation (**Fig. 7B**), whereas both hERG1-R531C and hERG1-K525C impaired the integrin-dependent phosphorylation of Tyr^{397} in FAK (**Fig. 7C**). We concluded that the signaling interaction between hERG1 and β1 integrin was similar in HEK and cancer cells. Finally, we studied the effects of disrupting the hERG1/β1 integrin complex in vivo, by using MDA-MB-231 breast cancer cells transfected with either hERG1, or hERG1-K525C, or hERG1-R531C. Cells were injected into either the right or left fourth breast of Severe Combined Immunodeficiency Disease (SCID) mice. The number of breasts displaying tumor masses, the median volume of the tumor masses, the presence of metastases in inguinal lymph nodes and lungs were determined 5 weeks after injection (**Fig. 7D** and **Fig. S9**). Although the growth of tumors formed from MDA-MB-231 cells was not affected by overexpression of either mutant, the percentage of mice with metastases in either the inguinal lymph nodes or in the lungs (**Fig. 7D** and **Fig. 7E**) was significantly decreased when the cells expressed hERG1-K525C. The percentage of metastatic area (**Fig. 7F**) and the number of metastatic clusters (**Fig. 7G**) was decreased in mice injected with MDA-MB-231 cells expressing hERG1-K525C, compared to those injected with cells expressing either wild-type hERG1 or hERG1-R531C. These results suggested that disrupting the

hERG1/β1 complex by overexpressing the hERG1-K525C mutant impaired the metastatic process.

DISCUSSION

We used co-immunoprecipitation experiments and FRET imaging to examine whether hERG1 and β1 integrin interacted directly on the plasma membrane. HS-FLIM-FRET showed an increase in the fast lifetime component in cells seeded and spread onto fibronectin. The high FRET efficiency suggests that the two proteins were in close proximity, within the upper limit of the CFP-YFP dynamic range of 7.3 nm (*43*). Whether proximity was caused by direct interaction or through the participation of other proteins in a multiprotein complex cannot be distinguished. The interaction was increased by cell adhesion on fibronectin. FRET experiments further demonstrated that hERG1 and β1 integrin interaction occurred only at the plasma membrane and not in cytosolic compartments. Moreover, co-immunoprecipitation experiments showed that hERG1 and β1 integrin interacted on the plasma membrane in a manner that did not require the cytoplasmic domains of these proteins. Nevertheless, because the hERG1 C-terminus regulates channel gating, an indirect contribution to the complex formation cannot be ruled out. Although we did not provide evidence excluding that the two proteins interact through their extracellular domains, we favor the conclusion that the interaction takes place largely through the transmembrane portions. First, it seems unlikely that the highly extended open conformation of the activated integrin (*44*) would interact substantially with the very short extracellular domain of hERG1. Second, the interaction between the transmembrane domains would seem more consistent with the gating-dependence of the complex formation and the competition between β1 integrin and KCNE1. The transmembrane domain of β1 integrin is a short α–helix, which also undergoes profound conformational changes upon integrin engagement (*45*), and could interact with the hERG1 domains that constitute the voltage sensor (*11*).

Instead, we found that assembly of this macromolecular complex depended on the channel's conformational state. We derived this conclusion by comparing the degree of complex formation of wild-type and mutant hERG1 channels. From the respective steady-state activation curves (*39, 40*) (**Fig. 5A and 5C**), hERG1 and hERG1-R531C should primarily reside in the closed (deactivated)

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state, whereas hERG1-K525C and the non-inactivating hERG1-S620T should spend more time in the open state. Because the latter two mutants associated poorly with β1 integrin, we concluded that complex formation was hindered when hERG1 was in the open state. Consistent with this notion, we observed impairment of complex formation in the presence of E4031 (**Fig. 4A**), which occupies the channel pore and thus maintains hERG1 in the open conformational state (*11*). Our interpretation is consistent with the results obtained with the non-conducting hERG1-G628S mutant, which shows impaired current flow but a greater ability to interact with β1 integrin than hERG1-K525C, hERG1-S620T, or wild-type hERG1 inhibited by E4031.

On the other hand, most of the conditions we tested tended to block the phosphorylation of Tyr^{397} in FAK (namely, auto-phosphorylation), which we used as an early marker of integrin-dependent downstream signaling. The common outcome of our treatments was a decrease in current flow through hERG1. E4031 blocked the channel pore; hERG1-G628S had a very low intrinsic channel conductance; hERG1-R531C had a very low open probability at the V_{rest} typical of our cells; hERG1-K525C, although generally active at V_{rest}, tended nonetheless to display lower whole-cell current amplitude than wild-type hERG1. The exception was hERG1-S620T, which generated higher whole-cell current amplitude than that of wild-type hERG1 (**Fig. 5A and 5B**).Consistently with our interpretation, phosphorylation of Tyr^{397} in FAK in cells expressing this mutant was similar to that in cells expressing wild-type hERG1.

We showed that these mechanisms operated in cancer cells. In all conditions in which I_{HERG1} was impaired, in vivo tumor growth was decreased, suggesting that channel activation regulated cell proliferation, possibly by controlling signaling pathways downstream of FAK activation (*42, 46*). In contrast, disrupting the hERG1/β1 integrin complex impaired the metastatic process of breast cancer cells. Therefore, the molecular association between hERG1 and β1 integrin appears to modulate the intracellular machinery related to cancer cell migration and invasiveness. Another biologically relevant finding was that hERG1/β1 integrin complex was not detected in cardiac myocytes, which suggests that the complex could be specific to tumor tissue. This difference could

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be caused by direct competition between β1 integrin and the hERG1 ancillary subunit KCNE1 in cardiac myocytes (**Fig. 1**).The competition between KCNE1 and β1 integrin for hERG1 binding suggests that integrins could substitute for KCNE1 as a binding partner in certain tissues. Nonetheless, current evidence does not exclude the possibility that KCNE1 also binds to β1 integrin.

Our present working hypothesis is that β1 integrin-dependent cell signaling leading to autophosphorylation of FAK is regulated by hERG1 activation and requires normal current flow through the ion channel, whereas the membrane macromolecular complex tends to recruit hERG1 channels residing in the non-conducting states. As suggested by single cell analysis (*16, 17*), hERG1 reaches maximal stimulation within a few minutes of cell adhesion onto fibronectin. It is possible that hERG1 activation occurring early during the process of cell adhesion has a role in stimulating FAK phosphorylation and the ensuing signaling pathways, whereas the late formation of the macromolecular complex progressively shifts the hERG1 channel population towards the non-conducting state. This process would turn off the early signaling cascade and favor late integrin-dependent cytoskeleton reorganization towards migration and invasion (*7-8, 46*). Hence, these results open the way for studies aimed at developing hERG1-related pharmacological compounds targeting different aspects of the neoplastic progression.

MATERIALS AND METHODS

Chemicals and antibodies

Unless otherwise indicated, chemicals and antibodies were purchased from Sigma Aldrich (Milan, Italy). Cell Lysis Buffer for protein extraction was from Cell Signaling Technology (#9803) and Protein A/G-Plus Agarose, for immunoprecipitation, was from Santa Cruz Biotechnology (sc-2003). The following antibodies were used for immunoprecipitation (IP) and WB, at the indicated concentrations. Monoclonal antibody (Mab)-hERG1 (DT-331, Dival Toscana Srl); IP: 5 µg/mg protein. Rabbit polyclonal antibody against hERG1 C-terminus (hERG1 CT pan pAb, Dival Toscana Srl DT-552); WB, 1:1000. Rabbit polyclonal antibody against β1 integrin C-terminus (RM12, Immunological Science); WB, 1:1000. Mab-β1 (TS2/16, Biolegend); IP, 5 µg/mg protein. Rabbit polyclonal antibody against GFP/YFP (Ab290, AbCam); WB, 1:1000. Rabbit polyclonal antibody against total FAK (SC-8312, Santa Cruz Biotechnology); IP, 1 µg/mg protein; WB, 1:1000. Rabbit monoclonal antibody against total FAK (EP695Y, AbCam); IP, 6 µg/mg protein; WB, 1:1000. Rabbit polyclonal antibody against p-Tyr^{397}FAK (BioSource International); WB, 1:1000. Mouse monoclonal antibody against KCNE1 (Abcam); WB, 1:500. Rabbit polyclonal antibody against human MHC class I (H-300) (sc-25619, Santa Cruz); immunohistochemistry, 1:100. Secondary antibodies for WB: Anti-rabbit IgG peroxidase antibody (whole molecule, A0545) 1:10000 and Anti-mouse IgG peroxidase antibody (whole molecule, A4416) 1:5000. The hERG1 inhibitor E4031 was used as previously described (*23*) at a final concentration of 40 µM, except for the patch-clamp experiments shown in Fig. 4A, where E4031 was used at 2 µM.

Plasmids

To produce the pECFP-hERG1 plasmid, the full hERG1 cDNA sequence was subcloned from pcDNA3.1(+)hERG1 into pECFP-N3 plasmid. Before cloning, to maintain the correct frame between ECFP and hERG1, two nucleotides were inserted between a Hind III recognition site and

the hERG1 start codon: a 500 bp PCR fragment of hERG1 cDNA was amplified from

pcDNA3.1(+)hERG1 plasmid using the forward primer **TAAGCTT**<u>**GG**</u>*ATG*CCGGTGCGGAGG,

with a Hind III recognition site (bold) at the 5' end of fragment, the hERG1 start codon (italics)

preceded by a mismatch of two nucleotides (underscored), and the reverse primer

GACCGCACCGACGACTCCCGGG. After denaturation, the DNA fragment was amplified using

PCR Platinum Supermix taq polymerase (Invitrogen), for 25 cycles (denaturation at 94°C for 30 s,

annealing at 50°C for 1 min, and extension at 72° C for 1 min). After cloning into a PCRII cloning

vector (Invitrogen), the amplified fragment was digested with HindIII and BstXI restriction

enzymes and inserted into pcDNA3.1(+)hERG1, to substitute the first 500 bp of hERG1 cDNA.

The modified hERG1 cDNA was then subcloned into pECFP-N3 after Hind III/ BamHI double

digestion. The pECFPhERG1 functionality was tested by WB and patch-clamp experiments.

The clone pCDNAhERG1ΔC+RD was generated according to Kupershmidt et al. (*35*). Briefly, a

315 bp PCR fragment, spanning nt 3052-3366 of hERG1 cDNA, was amplified from the

pCDNAhERG1 plasmid. The forward primer was

GGA***CTCGAG***CCCACCCCCAGCCTCCTCAACATCCC, with a XhoI recognition site (bold)

inserted at the 5' end. The reverse primer was

CTA**TCTAGA***CTA*CGGGGGCAGCTCCTCACACGCCATG, with a stop codon TAG (italics)

and a XbaI recognition site (bold) inserted at the 3' end of the fragment. The amplified DNA,

digested with XhoI and XbaI restriction enzymes, was inserted into XhoI/XbaI digested

pCDNAhERG1 plasmid, substituting the whole C-terminal domain (aa 699-1159). The obtained

clones were characterized by restriction pattern analysis and sequenced to verify the conservation of

reading frame.

WT β1 integrin (*47*), β1 integrin lacking the C-terminus (β1-extra) (*48*), and the C-terminus of β1

integrin linked to the transmembrane and extracellular part of the interleukin 2 receptor (β1-cyto)

were cloned into the Yellow Fluorescent protein Venus (YFP)-N1 vector. To generate the pYFP-N1

vector, YFP cDNA was amplified with BamHI and NotI from the pCS2Venus vector (*49*), and

substituted to EGFP in the pEGFP-N1 vector (Clontech). The pECE-β1 plasmid containing the full length β1A cDNA was cut with HindIII and SNABI to obtain a fragment of around 2000 bp, and amplified (SNAB1 and BamHI) to generate a fragment of around 400bp. The two fragments were cloned into the pYFP-N1 plasmid cut on HindIII/BamHI. The fragments corresponding to the β1-extra and β1-cyto constructs were amplified and cloned into the pYFP-N1 vector cut with KpnI and EcoRI.

Cell culture and transfection.

Cells were routinely cultured at 37°C with 5% CO_2 in either DMEM (Euroclone) (HEK 293-ICLC, GD25 and MDA-MB-231 cells) or RPMI (Euroclone) (HCT116 and REH cells) supplemented with 10% fetal bovine serum (Euroclone). HEK cells expressing wild type hERG1 were as previously described (*23*). To prepare the other stably transfected cell lines, transfection was carried out with Lipofectamine 2000 (Invitrogen). Selection and further cell culture were performed in complete culture medium supplemented with geneticin (G418; Invitrogen), at 0.8 mg/ml for HEK cells, and 2.0 mg/ml for HCT116 and MDA-MB-231 cells. For HS-FLIM-FRET experiments, HEK cells were transiently transfected with both pECPFhERG1 and pEYFP-β1 integrin plasmids. GD25 cells were transiently co-transfected with the different β1 integrin-YFP constructs and hERG1. GD25-β1-TR cells were described in (*47*).

Preparation of cells for FRET, immunoprecipitation and patch-clamp experiments

Cells were harvested by detaching them with 5 mM (10 mM for GD25 cells) EDTA in PBS, and resuspended in DMEM plus heat inactivated BSA (Fraction V, Euroclone; 250 μg/ml). Next, cells were seeded onto dishes coated first with fibronectin (100 μg/ml in serum-free medium, at 37°C for 1 h), and then with BSA for 1h. FRET measurements started 15 min after cell seeding and

continued for at least 100 min, at 37 °C. When necessary (**Fig. 2B**), dishes coated with BSA only were also used.

RNA extraction, reverse transcription and RT-qPCR

RNA extraction and reverse transcription were performed as described (*50*). Amplification of KCNE1 was performed by RT-PCR with 2 μl of cDNA derived from cell lines and human heart RNA (Ambion®), using a commercially available master mix (Invitrogen). The primer sequences for KCNE1 were: 5'-TCCATTGGAGGAAGGCATTA-3' (forward primer) and 5'-CGCTGTGGTGTTAGACAGGA-3' (reverse primer). PCR was performed as follows: denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 30 s and a final extension cycle at 72°C for 10 min. The same primers were used for RQ-qPCR analysis on HEK and HCT116 cells transfected with the KCNE1 plasmid. The SYBR green fluorescent dye (Power SYBR® Green, PCR master mix, Applied Biosystems) method was applied. GAPDH gene was used as a standard reference, as in (*26*). Non-transfected cell lines were used for calibration.

Immunoprecipitation and immunoblotting.

Procedures on cell lines were performed as previously described (*23, 26*). Cardiac and cancer tissues were obtained by individual donors undergoing surgery. Leukemia samples were obtained from bone marrow aspirates of patients at the onset of the disease. Patients were treated at the Molinette Hospital (Turin, Italy), at the General Surgery of the Careggi Hospital, and at the Hemathology Department of the Careggi Hospital (Florence, Italy). Tissues were collected after informed written consent, and after approval of the local Ethics Committee. All procedures were carried out at 4 °C. Samples were homogenized in cold protein extraction buffer (1X Cell Lysis Buffer) and sonicated for 30 min. For immunoprecipiation, total lysates (1.5 mg for cell line extracts and 0.5 mg for primary samples) were subjected to a pre-clearing step by incubating them with protein A/G-Plus Agarose for two hours at 4°C. Thereafter, cell lysates were

immunoprecipitated with the appropriate antibody at the concentrations indicated in the paragraph "Chemicals and antibodies". Co-immunoprecipitation of $\beta 1$ integrin and hERG1 lysates was performed with Mab-$\beta 1$. Co-immunoprecipitation of hERG1 and $\beta 1$ integrin lysates was performed with Mab-hERG1. For both reactions, WBs were performed on immunoprecipitates and total lysates ("input") from the same sample with polyclonal antibodies against hERG1 or $\beta 1$. To determine phosphorylation (P) of Tyr^{397} in FAK, FAK was immunoprecipitated with a polyclonal antibody. WBs were performed on immunoprecipitates and total lysates with polyclonal antibodies against p-Tyr^{397} FAK, total FAK or tubulin.

Densitometric analysis.

Densitometric analysis was performed using ImageJ software (ImageJ 1.38, National Institutes of Health) on two different scans, after background subtraction, from at least three different experiments. When quantifying variations in hERG1-$\beta 1$ integrin interactions, the signal for the co-immunoprecipitated protein (for example: hERG1 when immunoprecipitating the $\beta 1$ integrin, or $\beta 1$ integrin when immunoprecipitating hERG1) was first divided by the signal of the protein used for immunoprecipitation (for example: $\beta 1$ integrin or hERG1), and then normalized to the signal of the corresponding protein in the total lysate (named "input hERG1", "input $\beta 1$"). The resulting value is indicated as "hERG1/$\beta 1$ integrin complex" throughout the manuscript and in the figures. FAK phosphorylation was measured in immunoprecipitates with FAK antibodies, first dividing the signal for Tyr^{397} of FAK by the signal of the immunoprecipitated FAK, and then normalizing this value to the amount of FAK in the total lysate (input FAK).

Flow cytometry.

Expression of wild-type and mutant hERG1 was assessed by FACS Canto flow cytometer as in (*28*). Acquisition and analysis were performed using FACS Diva software (BD Biosciences).

Values are expressed as Mean Fluorescence Intensity (MFI) fold changes compared to non-transfected HEK or MDA-MB-231 mock-transfected cells.

HS-FLIM-FRET experiments

We used a Multiphoton Microscope consisting of a Nikon TE2000-U inverted optical microscope, equipped with a Nikon PCM2000 Confocal Laser Scanning Unit, modified to allow the use of an Ultrafast Laser Source, equipped with a dichroic short-pass filter, suitable for multiphoton operation (650DCSPXR; Chroma Inc.). This system is made up of a mode locked Ti:Sapphire oscillator (Mira 900 F; Coherent Inc.) pumped by a frequency doubled Nd:YVO4 laser system at 532 nm, 5W (Verdi V5; Coherent Inc.). Emission is tunable in the range 700–980 nm, with typical pulse duration of 130 fs and 76 MHz repetition rate. The laser beam, after passing through a Faraday isolator (to prevent part of laser beam returning to the laser cavity), was directly coupled to the confocal scanning unit. The confocal head is supplied with two output fluorescence emission channels (*51*). For the measurements, the light emitted from the sample was sent to a spectrograph, coupled to a fast 16-channel photon counting photomultiplier, with a typical transit time spread of 150 ps (PML-16C; Becker & Hickl GmbH). A two-photon cut-off filter (BG39; Schott GmbH) was inserted in the fluorescence path in order to remove unwanted back-reflection of the laser light. Acquired signals were processed by the FLIM apparatus, which is based on Time-Correlated Single-Photon Counting (TCSPC) and allows the measurement of the fluorescence decay curve for each image pixel. Its core element is an SPC-830 module (Becker & Hickl GmbH) able to measure the arrival time delays of individual photons with respect to a synchronization pulse train, provided by the signal of a fast photodiode (PHD-100; Becker & Hickl GmbH) that detects a laser light reflection from the neutral density filter. Curve fitting with exponential decay curves to the measured data and displays of the results was performed by the software SPCImage (Becker & Hickl GmbH).

The specificity and sensitivity of the FLIM technique to detect FRET in cells was tested using HEK transiently co-transfected with β1 integrin-EYFP and hERG1-ECFP, either as single or as double transfectants (**Fig. S3**). The theoretical expressions used are described in Supplementary Materials and Methods, paragraph "Analysis of HS-FLIM-FRET data"**.** FLIM measurements can be affected by pH, temperature, previous prolonged exposure to radiation and ion concentration. Spectral control is thus essential, especially when performing FLIM experiments in living cells. The details of our procedure are given in the Supplementary Materials and Methods ("Analysis of HS-FLIM-FRET") and legends to **Figs. S4 and S5**.

FRET by acceptor photobleaching experiments

We used a custom-made confocal microscope equipped with a spinning disk unit, as previously described (*52*), and further detailed in the Supplementary Materials. FRET was detected by an increase of the donor signal on acceptor bleaching. From the ratio of the donor fluorescent emissions before and after acceptor photobleaching, the pixel-by-pixel value of FRET transfer was determined. The percentage of FRET events, which reflected the abundance of hERG1/β1 integrin complexes, was estimated as the number of pixels for which FRET was detected in relation to the number of pixels in which the β1 integrin signal was simultaneously recorded. A detailed description of data acquisition and analysis is also reported in the Supplementary Materials, in which flowcharts are included for image acquisition and processing steps. Data analysis was performed using MATLAB (The MathWorks). Experiments were performed on HEK cells expressing wild-type hERG1, hERG1-R531C, or hERG1-K525C cultured for 45 min on fibronectin-coated slides. Cells were fixed with 4% paraformaldehyde and labeled with Alexa-conjugated Mab-hERG1 and Mab-β1. Purified Mab-hERG1 and Mab-β1 were labeled using the Alexa Fluor 488 and 546 Protein Labeling Kits (Molecular Probes), respectively. Quantitative estimation of labeled antibodies was determined by electrophoresis in agarose gel and Coomassie Brilliant Blue staining.

Patch-clamp recording

I_{hERG1} was recorded in the whole-cell configuration of the patch-clamp technique, at room temperature (~25°C), with a Multiclamp 700A amplifier (Molecular Devices), as previously described (*53*). E4031 was used at 2 μM. Background currents and leakage were measured in the presence of E4031 and subtracted from the total current. Unless otherwise indicated, $[K^+]_o$ was 5 mM. hERG1-K525C currents were recorded in 2 mM extracellular $[K^+]$. The activation curves for hERG1, hERG1Δ2-370, hERG1ΔC+RD and hERG1-G628S were determined from peak tail currents (I_{tail}) at -120 mV (for 1.1 s), following 15 s conditioning potentials from 0 mV to -70 mV (10 mV steps, spaced 4 s apart). The holding potential (V_H) was 0 mV. The activation curves for wild-type hERG1, hERG1-K525C and hERG1-R531C were obtained following a stimulation protocol similar to that used by Zhang et al. (*39*). From a negative V_H (-80 to -120 mV), we applied 1 s test voltages (10 mV increments), every 15 s. Test voltages (V_t) varied from -60 to +40 mV (hERG1), from -70 to +50 mV (hERG1-K525C), and from -30 to +70 mV (hERG1-R531C). I_{tail} was elicited by repolarization to -50 mV (-80 for hERG1-K525C). The peak I_{tail} obtained at each V_t's was normalized to the maximum I_{tail} (I_{max}). The relation between I_{tail}/I_{max} and V_t was fit to a Boltzmann function with OriginPro 2015 (Origin Lab, Northampton, Massachusetts) software.

In vivo experiments

Experiments were performed at the Animal House of the University of Florence (CESAL). Mice were housed in filter-top cages with a 12-hour dark-light cycle, and had unlimited access to food and water. Procedures were conducted according to the laws for experiments on live animals (Directive 2010/63/EU), and approved by the Italian Ministry of Health (1279/2015-PR).

For subcutaneous xenografts, female nude mice (Harlan Laboratories) aged five to six weeks were injected subcutaneously in either flanks with 2×10^6 cells, resuspended in 50 μl ice-cold PBS and gently mixed with the same volume of ice-cold Matrigel. The following human cell lines were used:

HEK-hERG1, HEK-hERG1-G628S, HEK-hERG1-R531C and HEK-hERG1-K525C. Each experimental group comprised 3 mice. One group of mice injected with HEK-hERG1 was treated daily with E4031 (20 mg/Kg), injected into the peritoneum, for two weeks, starting the day after inoculum. The volume of tumor masses measured at the sacrifice (six weeks after inoculation) was calculated by applying the ellipsoid equation.

For breast orthotopic xenografts, female SCID mice (Harlan Laboratories) aged six weeks were injected in both fourth mammary fat pads (5 mice/group), with different cell types (2×10^6 cells per mouse): MDA-MB-231-hERG1, MDA-MB-231-hERG1-R531C or MDA-MB-231-hERG1-K525C. After injection, mice were monitored daily to ensure they did not show any signs of suffering or disease (such as weight loss, abdominal distension, or impaired movement). Five weeks after injection, mice were euthanized and tumor masses and tissues were collected and processed for histological analysis. The volume of tumor masses at the sacrifice was calculated by applying the following expression: $0.52 \times l_{min}^2 \times l_{max}$, where l_{min} and l_{max} are, respectively, the minimum and maximum measured length of the tumor mass. Hemotoxylin&Eosin (H&E) and immunostaining of lung metastases were performed as in (26). To quantify the metastatic burden images were acquired on a Leica DM 4000B microscope with a Leica DFC 320 camera using Leica QWin software (Leica Microsystems; Milan, Italy). The percentage of metastatic areas per microscopic field were determined as in (54). At least three fields per mouse lung and both lungs were examined.

Statistical analysis.

Unless otherwise indicated, data are given as mean values ± standard error of the mean (SEM), with n indicating the number of independent experiments. Statistical comparisons were performed with OriginPro 2015 and SAS 9.2 (SAS Institute, Cary, North Carolina) software. The normality of data distribution was checked with Kolmogorov-Smirnov (K-S) test. In the case of normal distributions, each data set was firstly checked for variances homogeneity, using the F-Test for equality of two variances and the Brown-Forsythe test for multiple comparisons. For data with unequal variances,

the Welch correction was applied. For comparisons between two groups of data, we used the Student's *t* test. A two-sample K-S test was performed to test whether two underlying probability distributions differed. For multiple comparisons, one-way ANOVA followed by Bonferroni's post-hoc test was performed to derive *P* values. As reported in Figure legends, in case of unequal variances ANOVA was followed by the Hochberg's GT2 post-hoc method. In the case of non-normal distributions, the non-parametric Kruskal-Wallis ANOVA followed by Dwass, Steel, Critchlow-Fligner (DSCF)'s post-hoc method was applied. The relevant P values are reported in the figure panels.

SUPPLEMENTARY MATERIALS.

Materials and Methods

Fig. S1. Characterization of HEK 293 and HCT116 cells transfected with KCNE1.

Fig. S2. Co-immunoprecipitation of HEK-hERG1 cells seeded on fibronectin

Fig. S3. Characterization of HEK CFPhERG1+β1YFP cells.

Fig. S4. Fluorescence decay of a donor control sample.

Fig. S5: Control experiments to validate FRET results.

Fig. S6. Protocol for image acquisition of HEK 293 cells.

Fig. S7. Background correction and FRET map calculation.

Fig. S8. FRET efficiency as a function of the ratio of donor to acceptor.

Fig. S9. Lymph node metastases in mice orthotopically injected with MDA-MB-231-hERG1 and MDA-MB-231-hERG1-R531C cells

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A.A. and A.B. designed and supervised the whole study; S.C. (Silvia Crescioli), F.Z., S.P., O.C., G.P., A.F. and S.M. performed co-IP and WB experiments; R.M. and S.C. (Stefano Coppola) performed and analyzed FRET experiments; L.G. M.D'A. and A.B. performed patch-clamp data; L.C. and V.M. prepared hERG1 ΔC+RD and YFP constructs; S.C. (Silvia Crescioli), F.Z. and A.F. prepared HEK-hERG1 mutants; A.A. prepared HCT116-KCNE1, HEK-KCNE1 and MDA-MB-231 cells transfected with hERG1 mutants; O.C. prepared slides for FRET experiments; M.F.B., D.R. and M.R. provided human heart samples; F.Z. and A.M. produced HEK cells for HS-FLIM-FRET experiments; S.P., M.S. and A.A. performed in vivo experiments; G.P. and S.P. analyzed mouse tissue samples; T.S and F.Q. revised FRET data; PD supervised co-IP data; A.A. and A.B. wrote the paper; and all authors commented on the manuscript.

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FIGURE LEGENDS

Fig. 1. HERG1 and β1 integrin associate in human cancer tissue but not cardiac tissue. (A)

Left panel: co-Immunoprecipitation (co-IP) of β1 and hERG1 from CRC, PC, AML and CML

samples. Right panel: co-IP of hERG1 and β1 from CRCs, PC and one CML sample. Bottom

panel: Western blots (WBs) of total lysates (input) from the same tumor samples for hERG1 or β1.

Representative of 3 independent experiments. Mab, monoclonal antibody. (**B**) co- IP of β1 and

hERG1 (left panel) and co-IP of hERG1 and β1 in human heart atria. Representative of at least 3

independent experiments. Differences in the molecular weights of hERG1 bands in the various

cancer tissues are due to differential post-translational processing (*57-58*).(**C**) Left panel: RT-PCR

of *KCNE1* and *gapdh* in a representative heart sample, two CRC cell lines (HCT8 and HCT116)

and one AML cell line (FLG 29.1). Lane 1: 100 bp standard (St); lane 2: negative control (neg.

control). Representative of 3 independent experiments. Right panel: WB of total protein lysates

from the same samples for KCNE1 and tubulin. (**D and E**) co-IP of β1 and hERG1 from HEK cells

(D) or HCT116 cells (E) transfected or not with KCNE1. Representative of 3 independent

experiments performed in each cell line; the corresponding densitometric results are given in the bar

graph. P values were calculated respect to HEK-hERG1 and HCT116-WT cells, respectively,

Student's *t* test.

Fig. 2. FRET shows close interaction of hERG1 and β1 integrin on the plasma membrane.

(**A**) Left: image in false colors corresponding to the τ_m shown in the lifetime histogram and

emission spectra (black curve), for a cell showing FRET. These spectra are compared to the

measured emission spectra for ECFP (cyan curve) and EYFP (yellow curve). Right: image in false

colors and related histogram corresponding to fast lifetime values τ_f (left) and to slow lifetime

values τ_s (right), for the same cell. Images are representative of 4 independent experiments, in

which 104 total cells were analyzed. **(B)** FRET measurements, expressed as donor mean lifetimes carried out on HEK-hERG1 cells seeded onto fibronectin (n = 14 cells, from 3 independent experiments) or BSA (n = 12 cells, from 3 independent experiments). P value was calculated respect to cells seeded on BSA, Student's *t* test. **(C)** Examples of lifetime images for a cell on fibronectin not showing FRET (top-left), for a cell on fibronectin showing FRET (bottom-left), for a cell on BSA not showing FRET (top-right), and for a cell on BSA showing FRET (bottom-right). **(D)** Representative raw images of donor (hERG1, left) and acceptor (β1, right) (before acceptor photobleaching) at two different focal planes, namely 'Bottom plasma membrane' and 'Z-slice plasma membrane' in HEK 293 cells. Scale bar: 10 μm. The intensity scale is displayed in the top right corner of each image. Each image is representative of 12 total images per experimental condition (WT and mutants). **E)** HERG1/β1 FRET efficiency histograms of 'bottom plasma membrane' (black solid line) images (n=6 images each sampling at least 5 cells obtained from two different transfection experiments) and 'Z-slice plasma membrane' (blue solid line) images (n=6 images each sampling at least 5 cells obtained from two different transfection experiments), filtered for β1 abundance (see Supplementary Materials on image processing protocol). Mean FRET efficiencies were 0.23 ± 0.05 (Bottom plasma membrane), and 0.24 ± 0.05 (Z-slice plasma membrane). The underlying distributions were not statistically different (two-sample K-S test).

Fig. 3. The cytoplasmic domains of hERG1 and β1 integrin are not necessary for the complex assembly. **(A)** HERG1 currents measured in HEK cells stably transfected with the indicated constructs. Representative of at least 4 cells per transfection condition analyzed in 3 independent experiments. **(B)** co-IP of hERG1 and β1 using the indicated antibodies from HEK-hERG1, HEK-hERG1Δ2-370 and HEK-hERG1ΔC+RD-expressing cells. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. Not statistically different, one-way ANOVA. **(C)** Scheme of the β1 integrin constructs used in these experiments. **(D)** IP of hERG1 from GD25 cells co-expressing the YFP-tagged constructs and hERG1. WBs were

performed with the YFP antibody or the hERG1 polyclonal antibody. The arrow on the left of the upper panel indicates the bands of β1 and β1-extra (lanes 2 and 3). Lanes 4 and 5 show two IPs of hERG1 from 0.5 or 1 mg of protein from β1-cyto-transfected GD25 cells. Bands corresponding to β1-cyto at approximately 80 KDa were not detected (dotted, two headed arrow). The panel on the right shows WBs using YFP antibody ("input YFP") or hERG1 polycloncal antibody ("input hERG1") on the same total cellular lysates from GD25 cells co-transfected with the indicated β1 integrin constructs and hERG1. In the top panel, the upper arrow indicates the expected molecular weight of YFP-conjugated β1 integrin and β1-extra (approximately 147 KDa). The lower arrow indicates the expected molecular weight of β1-cyto (approximately 80 KDa). All the data reported in D are representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P values for β1-cyto were calculated respect to β1-extra-hERG1 and β1-hERG1 cells, one-way ANOVA. **(E)** co- IP of β1 and hERG1 from GD25-β1-TR cells, which stably express the β1-extra construct. Blots are representative of two independent experiments.

Fig. 4. Role of hERG1 current in the macromolecular complex formation and surface expression of WT and mutant hERG1 proteins.

 (A) Effect of E4031 on whole-cell WT hERG1 currents in HEK-hERG1 cells. Current traces show the blocking effect on tail currents at -120 mV. Bars give the average hERG1 peak current densies in the presence or absence of 2 μM E4031 (n= 5 cells analyzed in 3 independent experiments). P value was calculated respect to hERG1 cells, Student's *t* test. **(B)** co- IP of β1 and hERG1 from HEK-hERG1 cells seeded onto fibronectin, in the absence or presence of 40 μM E4031. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P value was calculated respect to hERG1 cells, Student's *t* test. **(C)** Membrane abundance of the indicated hERG1 constructs measured in HEK cells by flow cytometry, and expressed as Mean Fluorescence Intensity (MFI). The MFI of mock-transfected HEK cells was set as 1. Representative of 4 independent experiments. Not statistically different, Kruskal-Wallis

ANOVA with DSCF's post hoc method. **(D)** Whole-cell hERG1 currents in HEK-hERG1 cells (upper traces) and HEK-hERG1 G628S cells (lower traces). Only the tail current at -120 mV are shown (conditioning potentials are given in Materials and Methods). Currents were measured in the presence of 40 mM extracellular K^+. Bars give the corresponding average peak current densities, for hERG1 and hERG1-G628S (n=8 cells analyzed in 3 independent experiments). P value was calculated respect to hERG1 cells, Student's *t* test. **(E)** co-IP of β1 and hERG1from HEK-hERG1 and HEK-hERG1 G628S cells seeded onto fibronectin. Representative of 5 independent experiments; the corresponding densitometric results are given in the bar graph. Not statistically different, Student's *t* test.

Fig. 5. **hERG1 gating regulates the hERG1/β1 complex formation.**

(A) Typical whole-cell current traces elicited in HEK cells stably transfected with either hERG1-K525C, hERG1-R531C or hERG1-S620T. Representative of at least 5 cells per transfection condition analyzed in 3 independent experiments. **(B)** Comparison of the maximal current densities of hERG1, hERG1-K525C, hERG1-R531C and hERG1-S620T expressing HEK cells. Data are average peak tail current densities calculated from at least 5 cells per transfection condition analyzed in 3 independent experiments, at test pulses of +40 mV, +50 mV, +70 mV and +60 mV for hERG1, hERG1-K525C and hERG1-R531C respectively. P values were calculated respect to hERG1 or hERG1-R531C cells, Kruskal-Wallis ANOVA with DSCF's post-hoc method. **(C)** Activation curves of hERG1, hERG1-K525C, and hERG1-R531C expressing HEK cells. Data points are normalized peak tail currents calculated from experiments as in (A). **(D)** co-IP of β1 and hERG1 from HEK 293 cells expressing hERG1, hERG1-K525C, hERG1-R531C seeded onto fibronectin. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P values were calculated respect to hERG1 cells, one-way ANOVA. **(E)** Same as (D) but for hERG1-S620T. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P value was calculated respect to

hERG1 cells, Student's *t* test with Welch correction. **(F)** Probability distributions of FRET

efficiency from confocal images of cells expressing β1 integrin and hERG1, hERG1-R531C or

hERG1-K525C (n=6 images each sampling at least 5 cells obtained from two different transfection

experiments), filtered for β1 abundance (see Supplementary Materials for details on image

processing). Mean FRET values are 0.24±0.06, 0.23±0.06 and 0.24±0.06 for hERG1, hERG1-

R531C and hERG1-K525C, respectively. No statistical difference was found between all the

distribution couples, as tested by two-sample K-S test ($P > 0.05$). **(G)** Percentage of FRET events

for hERG1, hERG1-R531C and hERG1-K525C (assessing complex formation between hERG1 and

β1). P value was calculated respect to hERG1 cells, one-way ANOVA with Hochberg's (GT2)

post-hoc method.

Fig. 6. **Phosphorylation of Tyr^{397} in FAK is regulated by hERG1 current.**

(A) Phosphorylation (P) of Tyr^{397} in FAK in HEK cells expressing the indicated hERG1 constructs

and treated or not with 40 μM E4031, measured after cell seeding on fibronectin. Representative of

4 independent experiments; the corresponding densitometric results are given in the bar graph. P

values were calculated respect to hERG1 cells, one-way ANOVA. hERG1-S620T vs hERG1 not

significant, Student's *t* test. **(C)** Volume of tumor masses obtained in nude mice after subcutaneous

injection of HEK 293 cells expressing the indicated hERG1 constructs. One group of mice injected

with HEK-hERG1 cells was treated by intraperitoneal injection of E4031. Data are relative to 6

tumor masses for group (3 mice per group). P values were calculated respect to mice injected with

hERG1 cells, one-way ANOVA.

Fig. 7. Effects of inhibiting hERG1 currents and hERG1/β1 complex formation on neoplastic

progression. (A) Expression of the indicated hERG1 constructs transfected in MDA-MB-231 cells,

measured as in Figure 4C. Bars show the average hERG1 MFI of 3 independent experiments. Not

statistically different, Kruskal-Wallis ANOVA with DSCF's post hoc method. **(B)** co-IP of β1 and

hERG1 from MDA-MB-231 cells expressing the indicated hERG1 constructs seeded onto fibronectin. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P values were reported respect to hERG1 and hERG1-R531C cells, one-way ANOVA. **(C)** Phosphorylation of Tyr^{397} in FAK in MDA-MB-231 cells expressing the indicated hERG1 constructs. Representative of 3 independent experiments; the corresponding densitometric results are given in the bar graph. P values were calculated respect to hERG1 cells, one-way ANOVA. **(D)** Table summarizing quantitative data obtained from the breast orthotopic xenograft model. **(E) Left** (Necropsy): photographs of lungs from representative animals in which MDA-MB-231 cells expressing the indicated hERG1 constructs were orthotopically injected in the breast. **Middle** (Lungs H&E): lung sections labeled with H&E from the same animal as in the left panels. **Right:** magnified images and sections immunostained with an antibody against human MHC I (hMHC I). Bars: 100 μm. **(F)** Percentage of metastatic area and **(G)** number of metastatic clusters per microscopic field in the lungs of mice injected with MDA-MB-231 cells expressing the indicated hERG1 constructs. Values are averages of measurements obtained in at least 3 different microscopic fields in both mouse lungs in 5 mice for each injected experimental group. P values were calculated respect to hERG1 and hERG1-R531C cells, one-way ANOVA.