1 A microtranslatome coordinately regulates sodium and potassium currents in 2 the heart

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31 ABBREVIATION LIST

32 AP: Action potential

33 APD: Action potential duration

34 Co-IP: Co-immunoprecipitation

35 ER: Endoplasmic reticulum

36 FISH: Fluorescence in-situ hybridization

37 IF: Immunofluorescence

38 IP: Immunoprecipitation

39 iPSC-CM: Cardiomyocyte derived from induced pluripotent stem cells

40 RBP: RNA binding protein

41 RNA-IP: RNA-immunoprecipitation

42 shRNA: Short hairpin RNA

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47 **ABSTRACT**

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49 Catastrophic arrhythmias and sudden cardiac death can occur with even a small imbalance
 50 between inward sodium currents and outward potassium currents, but mechanisms
 51 establishing this critical balance are not understood. Here, we show that mRNA transcripts
 52 encoding I_{Na} and I_{Kr} channels (*SCN5A* and *hERG*, respectively) are associated in defined
 53 complexes during protein translation. Using biochemical, electrophysiological and single-
 54 molecule fluorescence localization approaches, we find that roughly half the *hERG*
 55 translational complexes contain *SCN5A* transcripts. Moreover, the transcripts are regulated
 56 in a way that alters functional expression of both channels at the membrane. Association and
 57 coordinate regulation of transcripts in discrete “microtranslatomes” represents a new
 58 paradigm controlling electrical activity in heart and other excitable tissues.

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65 INTRODUCTION

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67 Signaling in excitable cells depends on the coordinated flow of inward and outward currents
68 through a defined ensemble of ion channel species. This is especially true in heart, where
69 the expression of many different ion channels controls the spread of excitation triggering the
70 concerted contraction of the ventricular myocardium. Even small perturbations in the
71 quantitative balance due to block or mutations affecting a single type of channel can initiate
72 or perpetuate arrhythmias and lead to sudden death. Repolarization is a particularly
73 vulnerable phase of the cardiac cycle, when imbalance of inward and outward currents can
74 prolong action potential duration and trigger arrhythmias such as Torsades de Pointes¹. The
75 genetic basis of such catastrophic arrhythmias is in many cases unknown; mechanisms
76 coordinating expression of multiple ion channels may represent novel disease targets.

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78 Cardiac I_{Kr} is critical for normal repolarization² and is a major target of acquired and
79 congenital long QT syndrome^{3,4}. I_{Kr} channels minimally comprise hERG1a and hERG1b
80 subunits^{5,6}, which associate cotranslationally⁷ and preferentially form heteromultimers⁸.
81 Underlying heteromultimerization is the cotranslational association of *hERG1a* and *1b* mRNA
82 transcripts⁹. Because current magnitude is greater in heteromeric hERG1a/1b vs. homomeric
83 hERG1a channels, and loss of hERG1b is pro-arrhythmic^{5,10}, the mechanism of
84 cotranslational assembly of hERG subunits is important in cardiac repolarization⁹.

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86 In this study we found that association of transcripts could occur not only between alternate
87 *hERG* transcripts encoded by a single gene locus, but also between transcripts encoding
88 entirely different ion channel types whose balance is critical to cardiac excitability. Indeed, we
89 show that *SCN5A*, encoding the cardiac Na_v1.5 sodium channel, associates with *hERG*
90 transcripts as demonstrated by co-immunoprecipitation of nascent protein in heterologous
91 expression systems, cardiomyocytes derived from human induced pluripotent stem cells, and
92 native human myocardium. Single-molecule fluorescent *in situ* hybridization (smFISH)

quantitatively reveals *hERG* and *SCN5A* transcript colocalization captured during protein translation. Targeting *hERG* transcripts for shRNA degradation coordinately reduces *SCN5A* transcript levels as well, along with native I_{Kr} and I_{Na} currents recorded from cardiomyocytes. Thus, cotranslational association and regulation of transcripts is a novel mechanism establishing and preserving a balance of I_{Kr} and I_{Na} in heart, where relative levels of these currents are critical for normal action potential production and coordinated electrical activity.

RESULTS

Copurification of *hERG 1a* and *SCN5A* transcripts with their encoded proteins

Using specific antibodies that target the N-terminus of hERG1a, we purified hERG1a protein from induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and human ventricle lysates and performed RT-PCR to identify associated transcripts ("RNA-IP"; Fig. 1a). As previously reported⁹, both *hERG1a* and *1b* transcripts co-immunoprecipitated with nascent hERG 1a protein. Surprisingly, *SCN5A* transcripts encoding Na_v1.5 channels also copurified with nascent hERG1a protein (Fig. 1b and Supplementary Fig. S1). The interaction appears specific since neither ryanodine receptor RyR2 nor inward rectifier channel Kir2.1 (*KCNJ2*) transcripts copurified as part of this complex. The counterpart experiment using anti-Na_v1.5 antibodies confirmed association of transcripts encoding hERG1a, hERG1b and Na_v1.5, but not RyR2 (Fig. 1b). Bead-only controls showed no signal, indicating specific interactions of antibodies with corresponding antigens. The association also occurred in HEK293 cells, where additional controls showed that the antibodies used did not interact nonspecifically with mRNA encoding the other ion channels or subunits (Supplementary Fig. S1). Interestingly, when lysates independently expressing hERG1a and Na_v1.5 were mixed, hERG1a antibodies copurified only *hERG1a* mRNA, and Nav1.5 antibodies copurified only *SCN5A* mRNA, indicating that association of the two mRNAs requires their co-expression *in situ*. In addition, the interaction between *hERG1a* and *SCN5A* does not require the presence of *hERG1b* (Supplementary Fig. S1). This experiment demonstrates that transcripts encoding hERG1a, hERG1b and Na_v1.5 physically interact within the cell and can be copurified using antibodies targeting either nascent hERG1a or Na_v1.5 proteins. Their association with either encoded protein implies the transcripts associate during protein translation, or *cotranslationally*.

hERG1a and *SCN5A* transcript distribution

To independently confirm *hERG1a* and *SCN5A* transcript association, we performed single-molecule fluorescence *in situ* hybridization (smFISH) experiments in iPSC-CMs (Fig. 2a). We

used a combination of short DNA oligonucleotides (20 nucleotides), each labelled with a single fluorophore, that bind in series on the target mRNA and collectively are detected as a single fluorescent spot¹¹ (see Methods). Probes for *hERG1a* and *SCN5A* mRNAs were designed with spectrally separable labels for simultaneous detection (Quasar 647 and 546 respectively; see Methods and Supplementary Fig. S2 for probe validation, and Table S1 for list of probes)¹². Punctate signal for each mRNA species appeared singly and in clusters (Fig. 1a, b). To evaluate mRNA copy number in each detected signal, we fitted the histogram of the total fluorescence intensity of smFISH signals with the sum of Gaussian functions and determined mean intensity of a single mRNA molecule for each species (Fig. 2b and Supplementary Fig. S3). We found that approximately 25% of detected molecules exist singly, whereas about 20% occupy clusters containing 6 or more transcripts (Fig. 2c). Both transcripts were observed throughout the cytoplasm with higher density within 5-10 μ m from the nucleus (Fig. 2a, d), consistent with the expected distribution of perinuclear endoplasmic reticulum where these mRNA molecules are translated into proteins. A *GAPDH* mRNA probe set served as a positive control for smFISH experiments (Stellaris[®] validated control). In contrast with signals observed for *hERG1a* and *SCN5A* transcripts, *GAPDH* transcript clustered less, with 50% found as single molecules and <5% in clusters of 6 or more transcripts (Fig. 2c). Moreover, *GAPDH* molecules distributed more homogeneously throughout the cytoplasm with higher density in the range of 10 to 20 μ m from the nucleus (Fig. 2d). We noted similar numbers of *hERG1a* and *SCN5A* transcripts per cell but fewer than those for *GAPDH* (Fig. 2e). Thus, numbers and spatial distribution of *hERG1a* and *SCN5A* transcripts can be simultaneously resolved. Further work will be required to elucidate the significance or possible physiological role of differently sized mRNA clusters.

***hERG1a* and *SCN5A* transcript expression levels correlate**

Although we observed a range in numbers of *hERG1a* and *SCN5A* mRNAs among iPSC-CMs (Fig. 2e), regression analysis revealed clear correlation in their expression levels within

a given cell (Fig. 3 and Supplementary Table S3). Plotted against each other, *hERG1a* and *SCN5A* mRNA numbers exhibited a coefficient of determination (R^2) of 0.57 ($P=0.00001$; 41 cells; Fig. 3a and b). In contrast, pairwise combinations of *hERG1a* and *RyR2*, *hERG1a* and *GAPDH*, or *SCN5A* and *GAPDH* exhibited much lower linear correlation ($R^2=0.22$, $P=0.017$; $R^2=0.18$, $P=0.15$; and $R^2=0.33$, $P=0.000134$ respectively; $n=26$, 13, and 28 cells respectively; Fig. 3c and d, Supplementary Fig. S5 a and b, and Supplementary Table S3). Spearman coefficients revealed similar results as Pearson coefficients, where significant correlation is observed only between *SCN5A* and *hERG1a* (Supplementary Table S3). These findings indicate a roughly constant ratio of *hERG1a* and *SCN5A* mRNA copies.

***hERG1a* and *SCN5A* transcripts colocalize**

To determine potential *hERG1a* and *SCN5A* transcript association using smFISH, we measured proximity between the two signals using the centroid position, scored from touching to 67% (1 pixel) overlap (Fig. 4a and b). To discern colocalization from random overlap, we calculated the expected number of particles that could associate based on chance only for the different association criteria. Two-tailed t tests with Bonferroni correction revealed association between *hERG1a* and *SCN5A* transcripts significantly greater than that expected by chance (see Methods; P values summarized in Supplementary Table S2; Fig. 4b). Approximately 25% of each transcript population was associated with the other (Fig. 4c). To test specificity of interaction between *hERG1a* and *SCN5A* transcripts, smFISH and pairwise comparisons were also performed with *RyR2* and *GAPDH* transcripts, which revealed no significant association (Fig. 4d and e; Supplementary Table S2). These results show that association of *hERG* and *SCN5A* transcripts demonstrated in lysates can also be visualized in iPSC-CMs *in situ*, and provide strong evidence for the existence of a discrete mRNA complex comprising *hERG1a* and *SCN5A* transcripts.

Discrete *hERG1a* and *SCN5A* cotranslational complexes

To further explore whether colocalized mRNAs were part of a translational complex, we combined smFISH with immunofluorescence using hERG1a antibodies. We observed close association between *hERG1a* and *SCN5A* mRNAs and hERG1a protein significantly greater than that expected by chance (Fig. 5a and b and Supplementary Fig. S6a and b). Interestingly, among the 16% of actively translated *hERG1a* mRNAs (i.e. those associated with hERG1a protein), 46% were also associated with *SCN5A* mRNAs (Fig. 5c), indicating a 3-fold enrichment of their association in translational complexes. Analysis of the distribution of colocalized molecules revealed that 70% are located close to the nucleus (within 10µm, Fig. 5d).

We monitored association of hERG1a protein and transcript in the presence of puromycin, which releases translating ribosomes from mRNAs¹³ (Fig. 6a). We observed no change due to puromycin in the total number of respective mRNAs detected per cell (Fig. 6b). As expected, puromycin reduced association between *hERG1a* mRNA and hERG1a protein (antibody) and the S6 ribosomal protein (Fig. 6c). In addition, triple colocalization of *hERG1a* and *SCN5A* transcripts and either hERG1a protein or the ribosomal subunit S6 was robustly reduced (Fig 6d). These findings further support the conclusion that *hERG1a* and *SCN5A* associate cotranslationally.

hERG1a and *SCN5A* mRNAs are coregulated

We previously demonstrated that targeted knockdown of either *hERG1a* or *1b* transcripts by specific short hairpin RNA (shRNA) caused a reduction of both transcripts not attributable to off-target effects in iPSC-CMs or in HEK293 cells⁹. To determine whether *hERG* and *SCN5A* transcripts are similarly subject to this co-knockdown effect, we evaluated expression levels by performing RT-qPCR experiments in iPSC-CM. We found that *hERG1a*, *hERG1b* and *SCN5A* expression levels were all reduced by about 50% upon *hERG1a* silencing compared to the effects of a scrambled shRNA (Fig. 7a, orange bars). *RYR2* transcript levels were

unaffected. We observed similar results using the specific *hERG1b* shRNA (Fig. 7a, blue bars). Expressed independently in HEK293 cells, only *hERG1a* mRNA was affected by the 1a shRNA, and only *hERG1b* was affected by the 1b shRNA (Fig. 7b). *SCN5A* was unaffected by either shRNA, indicating that the knockdown in iPSC-CMs was not due to off-target effects and levels of associated *hERG1a* and *SCN5A* are quantitatively coregulated. Similar results of approximately 40% co-knockdown of discrete *hERG1a* and *SCN5A* mRNA particles were obtained using smFISH (Supplementary Fig. S7). Even more than the total population of mRNA, the number of colocalized particles is decreased by approximately 55%, indicating that physically associated transcripts are subjected to co-knockdown (Fig. S7c). Together these results indicate a coordinated and quantitative regulation of mRNAs encoding a complement of ion channels.

***I_{Kr}* and *I_{Na}* are coregulated**

To assess functional consequences of transcript coregulation, we recorded effects of *hERG1b* silencing on native currents in iPSC-CMs. Fig. 7c shows the repolarizing current *I_{Kr}* in iPSC-CMs transfected with either *hERG1b* or scrambled shRNA. Steady state and peak tail *I_{Kr}* were decreased in *hERG1b*-silenced cells compared to cells transfected with scrambled shRNA (Fig. 7d). *I_{Kr}* reduction was the result of a decrease in *G_{max}* upon *hERG1b*-specific silencing with no modifications in the voltage dependence of activation (Fig. 7e and Supplementary Table S4). These results are in accordance to our previous studies reporting a reduction in *I_{Kr}* density upon *hERG1b*-specific silencing, and indicate that transcripts targeted by shRNA are those undergoing translation^{9,10}. To determine whether *hERG1b* silencing also affects translationally active *SCN5A*, we measured peak *I_{Na}* density in iPSC-CMs and detected significant reduction of about 60% when *hERG1b* was silenced, compared to control cells (Fig. 7f, g and h). Peak *G_{max}* was decreased but no alterations in voltage dependence of activation or inactivation were detected (Fig. 7h and Supplementary Tables S4 and S5). Late *I_{Na}*, measured as the current integral from 50 to 800 ms from the beginning of the pulse¹⁴, was similarly reduced in magnitude (Fig. 7i, j and k). This analysis indicates

that coregulation via co-knockdown results in quantitatively similar alteration of $I_{Na,late}$ and I_{Kr} , which operate together to regulate repolarization¹⁵. I_{to} , which does not regulate action potential duration in larger mammals¹⁶, is unaffected by *hERG1b* silencing (Fig. 8a, b, c and d), suggesting the coregulation of I_{Na} and I_{Kr} reflects their coherent participation in repolarization.

DISCUSSION

We have demonstrated using diverse and independent approaches the association and coregulation of transcripts encoding ion channels that regulate excitability in cardiomyocytes. By co-immunoprecipitating mRNA transcripts along with their nascent proteins, we have shown that *hERG* and *SCN5A* transcripts associate natively in human ventricular myocardium and iPSC-CMs as well as when heterologously expressed in HEK293 cells. Using smFISH together with immunofluorescence in iPSC-CMs, we demonstrate that the ratio of *hERG* and *SCN5A* transcripts is approximately 1:1 despite a range of pool sizes from roughly 5 to 200 molecules per cell. These transcripts colocalize about 25% of the time, but when considering only those *hERG* transcripts undergoing translation, nearly 50% are associated with *SCN5A*. When *hERG1a* or *hERG1b* transcripts are targeted by shRNA, *SCN5A* levels are reduced by about the same amount. Both peak and late I_{Na} are correspondingly reduced. Reflecting their coherent roles in the process of cardiac repolarization, the term “microtranslatome” captures the cotranslational properties of this discrete complex comprising functionally related mRNAs and their nascent proteins.

What is the functional role of cotranslational association of transcripts? Deutsch and colleagues showed that cotranslational interaction of nascent Kv1.3 N-termini facilitates proper tertiary and quaternary structure required for oligomerization^{17,18}. Cotranslational heteromeric association of *hERG1a* and *hERG1b* subunits ensures cardiac I_{Kr} has the appropriate biophysical properties and magnitude shaping the normal ventricular action potential. Coordinated protein translation of *different* channel types could control relative numbers of ion channels involved in electrical signaling events. Such a balance is critical during repolarization, when alterations in I_{Kr} or late I_{Na} are known to cause arrhythmias associated with long QT syndrome or Brugada syndrome^{19–21}. Indeed, during normal Phase 3 repolarization, non-equilibrium gating of sodium channels leads to recovery from inactivation and re-activation of currents substantially larger than the tiny steady-state late I_{Na} observed under voltage-clamp steps^{15,22}. Our observation of roughly equivalent *hERG1a* and *SCN5A*

mRNA levels squares with previous reports of fixed channel transcript ratios associated with certain identified crustacean neurons^{23,24}. Cotranslating mRNAs in a stoichiometric manner could buffer noise associated with transcription²⁵ and render a stable balance of channel protein underlying control of membrane potential.

These studies raise questions of the mechanism by which transcripts associate. Although hERG1a and hERG1b N-termini interact during translation⁷, association of transcripts does not rely on this interaction: alternate transcripts encoding the proteins interact even when translation of one of the proteins is prevented⁹. In principle, transcripts could associate via complementary base pairing or by tertiary structural interactions as ligand and receptor. Alternatively, they could be linked by one or more RNA binding proteins (RBPs). Because the association and coregulation observed in native heart can be reproduced in HEK293 cells, the same or similar mechanisms are at work in the two systems. More work will be required to discern among possible mechanisms, and to determine the time course with respect to transcription, nuclear export and cytosolic localization of interacting transcripts.

A mechanism involving RBPs is appealing because it comports with the idea of the “RNA regulon,” a term describing a complex of transcripts bound by one or more RBPs^{26,27}. RBPs in the yeast Puf family bind large collections of mRNAs to control their localization, stability, translation and decay^{28,29}. In mammalian systems, the Nova protein serves to coordinate expression of mRNAs encoding splicing proteins important in synaptic function³⁰. Presumably in both cases these proteins interact in multiple regulons (complexes) serving different or related roles. Mata and colleagues isolated individual mRNA species in yeast and showed they associate with other mRNAs encoding functionally related (but nonhomologous) proteins, along with mRNA encoding the RBP itself³¹. Moreover, these mRNAs encoded proteins that formed stable macromolecular complexes³². Taking it one step further, Cosker et al. showed that two mRNAs involved in cytoskeletal regulation bind the same RBP to form

a single RNA granule³³, possibly analogous to the microtranslatome regulating key elements of excitability in the heart reported here.

A comprehensive analysis of the microtranslatome's components will require RNA-seq at a level of multiplexing that ensures sufficient statistical power in the face of potentially reduced complexity of the RNA-IP samples. These efforts will necessarily be followed by validation through complementary approaches such as RNAi and smFISH to confirm their identity within the microtranslatome.

One of the more curious findings of our study is the coordinate knockdown of different mRNAs in the complex by shRNAs targeted to only one of the mRNA species. The mechanism by which multiple mRNA species may be simultaneously regulated is not clear. shRNAs silence gene expression by producing an antisense (guide) strand that directs the RNA-induced silencing complex (RISC) to cleave, or suppress translation of, the target mRNA^{34,35}. Since hERG shRNA has no off-target effect on *SCN5A* mRNA expressed heterologously in HEK293 cells, we assume there is insufficient complementarity for a direct action. Perhaps by proximity to RISC, translation of the nontargeted mRNA is also disrupted, but to our knowledge no current evidence is available to support this idea. A transcriptional feedback mechanism seems unlikely given that co-knockdown can occur with plasmids transiently expressed from engineered promoters and not integrated into the genome of HEK293 cells. It is also important to note that it is unknown whether *SCN5A* is the only sodium channel transcript coregulated by *hERG* knockdown. In principle, transcripts encoding other sodium channels implicated in late I_{Na} , such as Nav1.8^{36,37}, could also be affected, as could transcripts encoding auxiliary subunits associated with Nav1.5³⁸.

Whether disrupting the integrity of these complexes gives rise to some of the many arrhythmias not attributable to mutations in ion channel genes *per se* remains to be determined. Although the coregulation of inward I_{Na} and outward I_{Kr} shown in this study may

suggest a compensatory mechanism, in a previous study we showed that selective knockdown of *hERG1b* prolongs action potential duration and enhances variability, both cellular markers of proarrhythmia¹⁰. Perhaps in the absence of co-regulation the effects would be more deleterious. Jalife and colleagues have introduced the concept of the “channelosome,” a macromolecular protein complex mediating a physiological action. Interestingly, Nav1.5 and Kir2.1, which regulates resting and diastolic membrane potential, exhibit compensatory changes when the levels of either are genetically manipulated³⁹. In this case, the effect seems to be on stability of the nontargeted channel proteins, which form a complex together with SAP97, and not on mRNA levels⁴⁰. We do not yet know whether the complex of transcripts we have studied encodes a similarly stable macromolecular complex, or perhaps ensures appropriate ratios of channels distributed independently at the membrane. Based on current evidence, we propose that the microtranslatome of associated transcripts is a novel mechanism governing the quantitative expression of multiple ion channel types and thus the balance of excitability in the cardiomyocyte.

METHODS

Cell culture, plasmids and transfection

HEK293 cells were cultured under standard conditions (37°C, 5% CO₂) in DMEM medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco). iPSC-CM (iCell®, Cellular Dynamics International) were plated and cultured following manufacturer's instructions. ShRNA sequences specific for hERG1a 5'-GCGCAGCGGCTTGCTCAACTCCACCTCGG-3' and its control 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3' were provided by Origene into a pGFP-V-RS vector. shRNA specific for hERG1b 5'-CCACAACCACCCTGGCTTCAT-3' and its respective control were purchased from Sigma-Aldrich. For heterologous expression, hERG1a (NM_000238) and hERG1b (NM_172057) sequences were cloned into pcDNA3.1. Transient transfections were performed using 2.5 µl/ml Lipofectamin™ 2000 (Thermofisher) with 2 µg/ml plasmid. Cells were collected for further analysis 48h after transfection. When needed, a second transfection was performed 24h after the first one with either hERG1a or hERG1b shRNA and the corresponding scrambled shRNA as a control. Cells were then collected for experiments 48h after last transfection.

Antibodies

Rabbit anti-hERG1a (#12889 from Cell Signaling, 1:100), rabbit anti-hERG1b (#ALX-215-051 from Enzo, 1:100), rabbit anti-pan hERG (#ALX-215-049 from Enzo, 1:3000), rabbit anti Na_v1.5 (#ASC-005 from Alomone or #D9J7S from Cell signaling, 1:500), were used for immunofluorescence, western blot or RNA-IP experiments. Alexa 647 goat anti-rabbit, Alexa 488 goat anti-rabbit or Alexa 488 donkey anti-mouse were employed for indirect immunofluorescence or immunoblotting experiments (Thermofisher; 1:1000).

RNA isolation and semi-quantitative real-time PCR

RNA isolation and purification were achieved using TriZol reagent (Life Technologies) and RNeasy Mini Kit (Qiagen). RT-qPCR experiments were performed using a TaqMan Gene

Expression Assay (Life Technologies) and mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ cycle threshold method. All data were normalized to mRNA level of β -actin housekeeping genes. Because iPSC-CMs are subject to inherent biological variability, we used a standardization procedure to normalize the independent biological replicates as previously described⁴¹. Briefly, a log transformation of the normalized relative expression gene level was performed, followed by mean centering and autoscaling of the data set. Results are expressed as average and 95% confidence intervals. Primers were purchased from Invitrogen (*hERG1a*: Hs00165120_m1; *hERG1b*: Hs04234675_m1; *SCN5A*: Hs00165693_m1; *RYR2*: Hs00892883_m1; and β -actin: Hs01060665_g1).

Immunofluorescence

For immunofluorescence studies, iPSC-CMs were grown on gelatin-coated coverslips, rinsed in PBS three times and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Following fixation, cells were incubated 1h at room temperature with a solution containing 0.5% triton 100X for permeabilization and 1% bovine serum albumin along with 10% serums (secondary antibodies species) diluted in PBS to saturate samples and limit nonspecific binding. Cells were then processed for indirect immunofluorescence using a combination of primary and secondary antibodies (see antibodies section above). Cells were washed three times with PBS, incubated with DAPI to counterstain nuclei and mounted with Vectafield mounting medium.

Single-molecule fluorescence *in situ* hybridization (smFISH)

FISH was performed using Stellaris® probe sets, which comprised up to 48 oligonucleotides designed to selectively bind in series the targeted transcripts. Probes were designed using the Stellaris™ Probe Designer by LGC Biosearch Technologies with the following parameters: masking level: 5, oligo length: 20 nucleotides, and minimum spacing length: 2 nucleotides. Oligonucleotides were labeled with TAMRA or Quasar® 670 dyes for detection of *SCN5A* and *hERG* respectively. 48 oligonucleotides were designed for *SCN5A*, *RyR2* and

GAPDH and 35 for the specific N-terminal sequence of *hERG1a*. Sequences for all probes are provided in Supplementary Table 1. FISH was performed on iPSC-CMs according the manufacturer's protocol. Briefly, fixation was performed by adding paraformaldehyde to a final concentration of 4% (32% solution, EM grade; Electron Microscopy Science) followed by a hybridization step for at least 4h at 37°C in a buffer containing a final concentration of 125 nM probes and 10% formamide (Stellaris hybridization buffer). Cells were washed for 30 min (Stellaris washing buffer A) before incubation for 30 min at 37°C with DAPI to counterstain the nuclei. A final washing step was performed (Stellaris washing buffer B) and coverglasses were mounted onto the slide with Vectashield mounting medium.

Digital images were acquired using a 63X objective on a Leica DMi8 AFC Inverted wide-field fluorescence microscope. Z-sections were acquired at 200 nm intervals. Image pixel size: XY, 106.3 nm. Image post-treatments were performed using ImageJ software (NIH). Briefly, a maximum projection was performed before background subtraction and images were filtered using a Gaussian blur filter to improve the signal/noise ratio and facilitate spot detection. Spot detection and colocalization was performed using the plugin ComDet on ImageJ^{42,43}.

FISHQUANT was used as a second method for spot detection and gave similar values. Briefly, background was subtracted using a Laplacian of Gaussian (LoG) and spots were fit to a three-dimensional (3D) Gaussian to determine the coordinates of the mRNA molecules. Intensity and width of the 3D Gaussian were thresholded to exclude non-specific signal^{11,12}. To evaluate the number of mRNA molecules, the total fluorescence intensity of smFISH signals was fitted with the sum of Gaussian functions (see equation below) to determine the mean intensity of a single mRNA.

$$y = y_0 + \frac{A}{w\sqrt{\pi}} e^{-2\left(\frac{x-x_c}{w}\right)^2}$$

Statistical analysis of smFISH and IF

For the purpose of our statistical calculations, we assumed that the protein and mRNA signals were circular. The following formulas were used to calculate the expected number of mRNAs (E_m) that would interact based on chance alone for each association criteria:

$$E_m = \frac{N_{m1}N_{m2}(2\pi r^2 - I)}{A}$$

where N_{m1} is the total number of mRNA in one channel, N_{m2} is the total number of mRNA in the second channel, r is the average radius of mRNA spots (in nm), I is the intersection between particles (nm^2), and A is the total area of the region analyzed (in nm^2). As the distance between particles is increased, the number of expected associated mRNAs will increase since more mRNAs will be considered associated. We used criteria with different stringency in the first set of experiments (from 1 pixel to 4 pixels distance between spots) and considered the 2 pixels distance between spots physiologically relevant for triple association analysis and co-knockdown experiments.

To test the significance of triple associations between hERG1a mRNA, SCN5A mRNA and hERG1a protein, the following formula was used:

$$E_p = \frac{N_p E_m (\pi r^2 - I)}{A}$$

where N_p is the total number of proteins, E_m is the expected number of mRNA that would interact based on chance alone as calculated above. For each association criteria, the intersection between particles was calculated using the following equation:

$$I = 2r^2 \cos^{-1}\left(\frac{d}{2r}\right) - \frac{1}{2}d(\sqrt{4r^2 - d^2})$$

Correlation analysis

mRNA numbers were plotted against each other from different combinations of smFISH signals as scatter plots. Then Pearson's and Spearman's correlation coefficients were evaluated to assess correlation between considered mRNA species. The following equation was used to calculate Pearson's coefficient R and determine the coefficient of determination R^2 from the mRNA pairs x_i, y_i :

$$R = \frac{Cov(x_i, y_i)}{\sigma_{x_i} - \sigma_{y_i}}$$

where $Cov(X_i, Y_i)$ is the covariance of the values and $\sigma_{x_i} - \sigma_{y_i}$ is the difference between the standard deviation of the values. Significance was determined using a F test.

The Spearman's coefficient ρ was determined on ranked values X_i and Y_i using the following equation:

$$\rho = \frac{Cov(X_i, Y_i)}{\sigma_{X_i} - \sigma_{Y_i}}$$

where $Cov(X_i, Y_i)$ is the covariance of the rank values and $\sigma_{X_i} - \sigma_{Y_i}$ is the difference between the standard deviation of the ranked values. Significance was determined using two-tailed probability test.

RNA-IP (RNA-immunoprecipitation)

Ribonucleoprotein (RNP) complexes were isolated with a RiboCluster Profiler TM RIP-Assay Kit (Medical & Biological Sciences) using protein-specific antibodies and Ab-immobilized A/G agarose beads. After formation of the RNP/beads complex, we used guanidine hydrochloride solution to dissociate beads from RNP complexes. Finally, target RNAs were analyzed using RT-PCR.

Electrophysiological measurements

Patch clamp under whole-cell configuration was used to record all ionic currents. I_{Kr} and $I_{Na,late}$ were recorded at physiological temperatures (37°C), while I_{Na} was recorded at room temperature (22°C) using an Axon 200B amplifier and Clampex Software (Molecular Devices). Glass pipettes with a resistance of 2.5 – 5 MΩ measured with physiological solutions (below) were pulled using an automatic P-97 Micropipette Puller system (Sutter Instruments).

To record steady state and tail I_{Kr} , cells were continuously perfused with an external solution containing (in mM): NaCl 150, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, Glucose 15, HEPES 15, Na-pyruvate 1, and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with an internal solution containing (in mM): NaCl 5, KCl 150, CaCl₂ 2, EGTA 5, HEPES 10, Mg-ATP 5, and the pH was adjusted to 7.3 with NaOH. The voltage protocol for I_{Kr} was completed at physiological temperature (37°C) and determined as an E-4031 (2μM) sensitive current. Cells were recorded using a holding potential of -50 mV, followed by a pulse at -40 mV to inactivate sodium channels, then 3-second depolarizing steps (from -50 to +30 mV in 10 mV increments) to activate hERG channels and finally to -40 mV for 6 seconds. Steady-state I_{Kr} was measured as the 5 ms average current at the end of the depolarizing steps. Tail currents were measured following the return to -40 mV.

To record I_{Na} , cells were perfused with an external solution containing (in mM): NaCl 50, Tetraethylammonium (TEA) methanesulfonate 90, CaCl₂ 2, MgCl₂ 1, Glucose 10, HEPES 10, Na-pyruvate 1, Nifedipine 10 μM, and pH adjusted to 7.4 with TEA-OH. Micropipettes were filled with an internal solution containing (in mM): NaCl 10, CaCl₂ 2, CsCl 135, EGTA 5, HEPES 10, Mg-ATP 5, and pH was adjusted to 7.3 with CsOH. I_{Na} activation was investigated by applying pulses between -140 and +20 mV in 10 mV increments from a holding potential of -120 mV. To measure inactivation of sodium channels,

conditioning pulses from -140 to +20 mV in 10 mV increments were applied from a holding potential of -120 mV following by a test pulse to -20 mV.

To record $I_{Na,late}$, cells were perfused with an external solution containing (in mM): 140, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 2, HEPES 5, Nifedipine 10 μ M, and pH was adjusted to 7.3 with NaOH. Pipette were filled with an internal solution containing (in mM): NaCl 5, CsCl 133, Mg-ATP 2, TEA 20, EGTA 10, HEPES 5, and pH was adjusted to 7.33 with CsOH. $I_{Na,late}$ was measured by applying an 800 ms single pulse to -30 mV from a holding potential of -120 mV. The mean current was measured at the last 200 ms of the pulse. An external solution containing 30 μ M TTX was perfused after the first pulse to determine if the current was due to the activity of sodium channels.

To record I_{to} , cells were continuously perfused with an external solution containing (in mM): NaCl 150, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Glucose 15, HEPES 15, Na-pyruvate 1, E4031 2, $CdCl_2$ 0.5 and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with an internal solution containing (in mM): NaCl 5, KCl 150, $CaCl_2$ 2, EGTA 5, HEPES 10, Mg-ATP 5, and the pH was adjusted to 7.3 with NaOH.

Both activation (for I_{Kr} , I_{to} and I_{Na}) and inactivation (for I_{Na}) were fitted to Boltzmann equations (Equations (1) and (2), respectively) and voltage dependence parameters were obtained.

$$I(V) = \frac{(V - V_{rev})G_{max}}{1 + e^{\frac{(V - V_{1/2})}{k}}} \quad (1)$$

$$I(V) = \frac{(I_{min} - I_{max}) + I_{max}}{1 + e^{\frac{(V - V_{max})}{k}}} \quad (2)$$

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Author Contributions

Experiments were conceived and designed by C.A.E, E.R.-P., F.L. and G.A.R. C.A.E. and M.B.J. carried out single molecule FISH experiments. F.L. and J.J.K. conducted RNA-IP and knock-down experiments. E.B.R-P. and D.K.J. performed electrophysiology experiments. C.A.E. and G.A.R. wrote the manuscript and all authors provided critical feedback to the final version.

Data Availability

The source data corresponding to Figures 1b, 2b, 2c, 2d, 2e, 3b, 3d, 4b, 4c, 4e, 5b, 5c, 5d, 6b, 6c, 6d, 7a, 7b, 7d, 7e, 7g, 7h, 7j, 7k, 8a, 8b, 8d and Supplementary Figures S1, S3a, S3b, S3c, S4a, S4b, S5a, S5b, S6b, S7b, S7c. Raw files images and other data supporting the findings of this study are available upon request.

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Figure 1: Complex of ion channel transcripts with nascent proteins. **a**, Scheme of the RNA-IP protocol in which channel-specific antibodies are used to pull down nascent proteins and associated transcripts. RNP: ribonucleoprotein. **b**, Lanes 1 and 2, RT-PCR products from input lysate of human left ventricle (LV), and iPSC-CM. Lanes 3-16 shows the corresponding RNA-IP's using an anti-hERG1a or anti-Nav1.5 antibodies; Lane 7 shows the control (+) and represents signal amplified from purified plasmid template. Similar results were obtained in at least 3 independent experiments. (N=5 for anti-hERG1a and N=3 for anti-Nav1.5 using human LV and iPSC-CMs).

Figure 2: Quantitative description of single *hERG1a* and *SCN5A* transcripts and their distribution in iPSC-CMs. **a**, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to the smFISH protocol. **b**, By fitting the intensity histogram of smFISH signals (n=2611 spots) to the sum of Gaussian functions (red line), the typical intensity corresponding to a single mRNA molecule (vertical dashed line) was extracted. **c**, The distribution of the number of mRNA molecules associated in clusters for each transcript evaluated by smFISH. **d**, Histogram showing the cytoplasmic distribution of mRNA signals with distance from the nucleus. **e**, The number of mRNAs detected per cell was plotted for *SCN5A*, *hERG1a* and *GAPDH* (lines represent mean $\pm$ SE).

Figure 3: *hERG1a* and *SCN5A* transcript expression levels correlate. **a**, Representative confocal images and enlargements of double smFISH experiments for *SCN5A* (red) and *hERG1a* (cyan) mRNAs. **b**, The number of mRNA molecules detected per cell in double smFISH experiments were plotted for *SCN5A* and *hERG1a* and the coefficient of determination R^2 was determined from the Pearson's correlation coefficient R ($n=41$ cells; $N=2$). **c**, Representative confocal images and enlargements of double smFISH experiments for *RyR2* (red) and *hERG1a* (cyan) mRNAs. **d**, The number of *hERG1a* mRNA was plotted against the number of *RYR2* mRNAs per cells and showed a low correlation in their expression ($n=26$ cells; $N=2$).

Figure 4: *hERG1a* and *SCN5a* transcript colocalization. **a**, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to smFISH showing the colocalization of *hERG1a* and *SCN5a* mRNAs. **b**, Comparison of the average number of associated *hERG1a* and *SCN5a* mRNAs particles observed vs. expected by chance using different overlap criteria illustrated (mean $\pm$ SE; $n=41$ cells; $N=2$). **c**, Diagram illustrating that the association of *hERG1a* and *SCN5a* mRNAs account for 24% and 23% of their total population respectively. **d**, Representative confocal images of smFISH for *hERG1a* and *RyR2* transcripts. **e**, Comparison of the average number of associated *hERG1a* and *RyR2* mRNAs particles observed vs. expected by chance using different overlap criteria (mean $\pm$ SE; $n=26$ cells; $N=2$).

Figure 5: Cotranslational association of *hERG1a* protein and *hERG1a* and *SCN5A* mRNAs. **a**, Representative confocal images and enlargement of iPSC-CMs subjected to immunofluorescence (IF) combined with smFISH protocol. Arrows indicate triply colocalized particles. **b**, The average number of particles comprising *hERG1a* and *SCN5A* mRNAs and *hERG1a* protein per cell compared to the expected number based on chance using a maximum distance of 2 pixels between center of mass (minimum 50% overlap; mean $\pm$ SE; n=13 cells, N=2). **c**, Histogram showing that 16% of *hERG1a* mRNA associate with *hERG1a* protein (actively translated population); of that percentage, 46% also interact with *SCN5A* transcripts.(mean $\pm$ SE; n=13 cells; N=2) **d**, Histogram showing the distribution of colocalized mRNA spots through the cytoplasm and from the nucleus revealing that RNP complexes are mostly localized within 10 μm from the nucleus. In the top right corner, representative examples of colocalized spots (yellow circles) and analysis of distance from the nucleus (white dashed arrows).

Figure 6: Distribution and association of *hERG1a* and *SCN5A* transcripts under puromycin treatment in iPSC-CMs. **a**, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to immunofluorescence combined with smFISH for control cells (left panel) or cells treated with 100 μ M puromycin for 15 min (right panel). **b**, The number of mRNAs detected per cell was plotted for *SCN5A* and *hERG1a* in the presence of puromycin and compared to control cells (lines represent mean $\pm$ SE). **c**, Histogram showing the reduction of association between *hERG1a* mRNA and *hERG1a* protein after puromycin treatment compared to non-treated cells (mean $\pm$ SE). **d**, Histogram showing that the % of triply colocalized particles (*hERG1a* protein or the ribosomal subunit S6 associated with both *hERG1a* and *SCN5A* mRNAs) is decreased upon puromycin treatment (mean $\pm$ SE).

Figure 7: Co-knockdown of I_{Kr} and I_{Na} by hERG transcript-specific shRNA. **a**, Effects of *hERG1a* or *hERG1b* silencing on channel mRNA expression levels detected by RT-qPCR (mean $\pm$ 95% CI) in iPSC-CMs. A non-targeting shRNA (scrambled shRNA) is used as a control. **b**, Effects of specific *hERG1a* or *hERG1b* silencing on ion channel mRNAs expressed alone in HEK293 cells. **c**, Representative family of traces show I_{Kr} in presence of control (upper) or hERG1b shRNA (lower). **d**, Summary of steady-state current density vs. test potential shows effect of hERG1b shRNA (mean $\pm$ SE). **e**, Effects of 1b shRNA on peak tail current vs. pre-pulse potential (mean $\pm$ SE). **f**, Representative family of traces recorded from iPSC-CMs showing effects of hERG1b-specific shRNA compared to control shRNA on peak I_{Na} . **g**, Summary current-voltage plot of peak I_{Na} vs. test potential (mean $\pm$ SE). **h**, Summary conductance (G)-voltage plot based on data from **g** (mean $\pm$ SE). **i**, Late sodium current representative trace in control and 1b shRNA-transfected cells, measured by applying a single pulse protocol of 800 ms. **j**, Summary statistics of peak I_{Na} showed a decrease upon transfection with hERG1b shRNA (mean $\pm$ SE). **k**, Late I_{Na} measured as the integral from 50 to 800 ms from the beginning of the pulse showed a decrease upon transfection with hERG1b shRNA (mean $\pm$ SE).

Figure 8: Effects of hERG1b silencing on I_{to} and $K_{V4.2}$ channels in iPSC-CMs. **a**, Effects of *hERG1a* or *hERG1b* silencing on $K_{V4.2}$ channel mRNA expression levels detected by RT-qPCR (mean $\pm$ 95%CI) in iPSC-CMs. A non-targeting shRNA (scrambled shRNA) is used as a control. **b**, Effects of specific *hERG1a* or *hERG1b* silencing on $K_{V4.2}$ channel mRNAs expressed alone in HEK293 cells. **c**, Representative family of traces show I_{to} in presence of control (upper) or hERG1b shRNA (lower). **d**, Summary of steady-state current density vs. test potential shows effect of hERG1b shRNA (mean $\pm$ SE).

Supplementary

Table S1

hERG1a probes	SCN5A probes	RyR2 probes
caagactggactgcgggc	cccgaggtaataggaagttt	tccattttccacatcaact
agcactaggcttcgggtg	tagagatctggcagcttttt	acatccaatttttcttgga
caggaaggtgttctgcgg	agtcttttgggtgctataga	tgtagatcccattctcac
acttcgggatgatggtgt	gaagatggtcttgcctttat	catgtcgggatctcgacaaa
aacttacggctctggccc	ggactgaggacatacaaggc	caaggcccttataaatcta
ccgagcgttggcgcgatgat	gtgtactcgacatacttgggt	gaagtagccaatgagatcct
agatgacggcgagttct	gaccagagactcaaagggtgt	tctgttctgttcttctcat
cagaagccgtcgttgacag	ccgaaggaaagtgaacgcgt	agtcgaggagccagaaaaat
cgagtagccgcacagctc	aatcacactaaagtccagcc	acatccagaaccttgtgatt
gcagggtcgtgcatcac	attcagttgtgtatgccatg	tggcaaacacagagtgcga
cgtgcaggaagtgcagg	tcgctaaggctgagacattg	tcacagatgagatgctgggt
cagtgcctgcgcgatctg	ccaggcaaaggaatcgaagc	atggttcacaagacgtgtct
gatttccactttcgctc	caggcggaagagtgaagaa	gatgaaggccatcaaatcca
cccatcttccggtagaa	tcagtaaacgggtccatgac	ttggtgagcttacagtacga
ccacagacataggaagc	tgtgttagtacgatgcaca	aggtgtccattaattcgga
ctcgttcttcacgggcac	tcctcgaattcacttgcac	agttcttcttgcctgtact
aacatgatgacagcccca	gggcaatgatcttgaaggtc	acttgcagctggtaattct
ccacctcgaaattgagga	tggaaagtagtaggggtc	tatgtgcattttctgccaac
accatgtccttccatc	gatgacgatgatgtgtcga	ggaacaaggcgaggatttct
gtggttgggtcatgagc	ccgatgatcttgatgagtg	cacggaagattcggaacctt
cttcaggcggaaaggtctt	gaacacgatgatggctagca	gtgtgaacatcatggtgtgt
ctgtcacttctgcagggt	agtagttcttccaaagagc	ctgacatccttccaaagtt
tgccacgtggtgtccat	aggttgccaatgaccataac	atggtaatatccctgtttgt
gtcggggttggagctgtg	atgaatgtctgaaccagct	cagtgtgtctgttctgagaa
ctttctcggagcgctc	cactgctgagtaggatcatg	tttctgggcataatctttg
catggcctcgaatgctgc	ctaggtagatgtcctcgaag	ggctttgttcttcaagcaa
tgcgcagtgggtgcatgg	caagcagaaccttgatggtc	tacgtggacgtttgcatcaa
gtggagttgagcaagccg	atctccagcacgaagacata	agtcaaaagcctgtgtcatac
cacgaggtcggagtccga	agtacttcttgaagccgtag	tggcaatgaatgtgagcagc
tgctaattggtgcggtagc	aatcgtgacagagctctcag	ggactttagcttgtgcaaa
ttgagggtgatttgggga	cgaggaggacgttcatgatg	gagagaggcatcacattctt
gcccttgagggtccacaaa	tgttcacgatggtgtagttc	ttcttgtgctcactcttgaa
gcgaagccaagaaggggt	agttcaaggactcacactgg	ttcgagacacatctacctt
gatctcacggtcactggt	ccacgttgtcaaagttgact	gggacaatgtactctgttgt
ctcctttatcttaggtgc	gctgcatacataatgtccat	gcttcaaaaactgggggagg
	tcctctgtcatgaagatgtc	acacactggggtcaatcaac
	catggcattgtagtacttct	ttgatctgttcttgagggtg
	aatatgaagccctgttactt	agacgccctctaattgttaa
	catcatggtcaccatattca	agtactctcaactggtttt
	cagcttgacaatacactcgc	gggtcttcaatgacagactc
	ttggtgaagtagtagtggtg	ctccgaatgagcaatctcac
	tggagaggatgacaaccacg	atggacctaattctgatggc
	aagagcgtcggggagaagaa	tgaacctcaatcccatagac
	catcatgagggcaagagca	ctgtgcaaagggtaccgattg
	acatgacgaggaagagcagc	agagaggagcacatcttgtt
	ccatgccaagatggagtag	gaacagccctagaagctct
	cccacttgacataagcgaag	aaacttctatggaatccgc
	taggagatcttgatgggtt	ttctgagtaagtgtgcatc

Table S1: List of probes used in smFISH experiments. The probes were designed using Stellaris® probe Designer software with the following parameters: 18 to 20 nucleotides oligo length, a masking level of 5, a minimum spacing length of 2 nucleotides and a maximum number of probes of 48. Due to the length of the N-terminal specific sequence for *hERG1a* mRNA, the number of probes used to detect *hERG1a* is limited to 35.

Table S2

		400 nm Touching	300 nm 20% overlap	200 nm 45% overlap	100 nm 67% overlap	
hERG1a/SCN5A (n=41 cells)	Expected by chance Measured P value	8.10 ± 0.87 16.68 ± 1.70 1.57E-9 (***)	5.56 ± 0.61 13.34 ± 1.38 4.71E-9 (***)	3.20 ± 0.34 11.12 ± 1.28 3.49E-10 (***)	0.40 ± 0.04 5.73 ± 0.82 4.51E-8 (***)	Significance after Bonferroni's correction: * P≤0.00125; ** P≤0.000122; *** P≤0.0000244
hERG1a/RyR2 (n=26 cells)	Expected by chance Measured P value	4.92 ± 0.66 5.92 ± 0.36 0.16 (ns)	4.03 ± 0.54 4.96 ± 0.30 0.16 (ns)	2.88 ± 0.39 3.69 ± 0.26 0.14 (ns)	1.74 ± 0.23 2.04 ± 0.24 0.38 (ns)	Significance after Bonferroni's correction: * P≤0.00197; ** P≤0.000193; ***P≤0.0000385
hERG1a/GAPDH (n=13 cells)	Expected by chance Measured P value	14.87 ± 2.65 13.54 ± 1.68 0.53 (ns)	7.47 ± 1.33 8.46 ± 1.15 0.20 (ns)	5.48 ± 1.23 5.24 ± 0.72 0.19 (ns)	0.54 ± 0.10 0.92 ± 0.15 0.24 (ns)	Significance with Bonferroni correction: * P≤0.0039; ** P≤0.000386; ***P≤0.000077
SCN5A/GAPDH (n=28 cells)	Expected by chance Measured P value	17.31 ± 1.71 15.92 ± 1.64 0.16 (ns)	12.73 ± 1.25 9.92 ± 1.04 0.002 (ns)	6.82 ± 0.67 5.32 ± 0.60 0.0006 (*)	0.92 ± 0.09 1.62 ± 0.28 0.012 (ns)	Significance after Bonferroni's correction: * P≤0.0013; ** P≤0.000186; *** P≤0.0000275

Table S2: Summary of colocalization analysis performed in iPSC-CMs for different association criteria. Comparison of the average number of mRNAs particles observed to be associated and the expected number based on chance alone using centroid positions and different association criteria (from touching to 67% overlap). The significance is tested with a paired t-test Bonferroni's correction. The number of *hERG1a* and *SCN5A* mRNAs observed to be associated is significantly above that expected by chance alone for all association criteria tested while no significant differences are observed for *hERG1a/RyR2*, *hERG1a/GAPDH* and *SCN5A/GAPDH* associations.

Table S3

		hERG1a/SCN5A (41 cells)	hERG1a/RyR2 (26 cells)	hERG1a/GAPDH (13 cells)	SCN5A/GAPDH (28 cells)
Pearson's test	Correlation coefficient R	0.7546	0.4654	0.4197	0.4808
	Coefficient of determination R ²	0.56943	0.2166	0.1761	0.2315
	P value	0.00001 (***)	0.01658 (*)	0.153373 (ns)	0.0096 (*)
	Slope of linear regression line	0.6844	0.3732	0.2662	1.4651
	Significance after Bonferroni correction	* P<0.038 ** P<0.0076 *** P<0.00076	* P<0.024 ** P<0.0049 *** P<0.00049	* P<0.023 ** P<0.0046 *** P<0.00046	* P<0.025 ** P<0.005 *** P<0.0005
Spearman's test	Correlation coefficient p	0.7449	0.3224	0.4890	0.3692
	P value	0 (***)	0.1084 (ns)	0.08991 (*)	0.05315 (ns)
	Significance after Bonferroni correction	* P<0.019 ** P<0.0039 *** P<0.00039	* P<0.0055 ** P<0.0011 *** P<0.00011	* P<0.013 ** P<0.0027 *** P<0.00027	* P<0.0061 ** P<0.0012 *** P<0.00012

Table S3: Summary of correlation analysis performed in iPSC-CMs. The linear correlation between the different combination of mRNAs was evaluated using the Pearson correlation coefficient. Because the Pearson coefficient is highly sensitive to outliers and only assess linear correlation, the Spearman's correlation coefficient was also calculated. Both tests revealed a significant correlation between hERG1a and SCN5A mRNAs and no significant correlation for *hERG1a/RyR2*, *hERG1a/GAPDH* and *SCN5A/GAPDH* pairs. Levels of significance were adjusted with a Bonferroni correction taking into account correlation coefficients and either linear correlation or non-linear correlation for Pearson's and Spearman's test respectively.

Table S4 and S5

Condition	$G_{\max}$ (nS/pF) or $I_{\max}$ (pA/pF)	$V_{1/2}$ (mV)	k (mV)	V_{rev} (mV)	n
Activation					
Control	1.22 ± 0.1	-45.9 ± 1.1	-4.7 ± 0.4	36.5 ± 3.0	16
1b shRNA	0.73 ± 0.1	-45.7 ± 1.6	-4.8 ± 0.5	31.7 ± 3.2	9
Inactivation					
Control	-54.1 ± 7.7	-89.8 ± 1.3	7.2 ± 0.3		16
1b shRNA	-33.2 ± 9.7	-88.6 ± 1.9	6.8 ± 0.5		9

Table S4: Voltage dependence of activation and inactivation parameters for the sodium channels in cells transfected with a control shRNA or a hERG1b specific shRNA. Parameters were obtained after fitting to a Boltzmann equation activation and inactivation data.

Condition	$I_{\max \text{ peak-tail}}$ (pA/pF)	$V_{1/2}$ (mV)	k (mV)	n
Control	0.50 ± 0.01	-26.0 ± 0.5	5.6 ± 0.5	5
1b shRNA	0.21 ± 0.03	-23.1 ± 4.5	7.1 ± 5.3	4

Table S5: Voltage dependence of activation of hERG channels in cells transfected with a control shRNA or a hERG1b specific shRNA. Parameters were obtained by fitting the experimental data of the I-V curve of the peak tail I_{Kr} to a Boltzmann equation.

Figure S1: Complete RNA-IP from Figure 1. Lanes 1-6, RT-PCR products from input lysate of human left ventricle (LV), iPSC-CM, and HEK293 cells expressing: *hERG1a*; *SCN5A*; *hERG1a* plus *SCN5A*; and *hERG1a* plus *hERG1b* and *SCN5A*. Lane 7 shows RT-PCR product from lysates independently expressing *hERG1a* and *SCN5A*, mixed. Lanes 8-14 shows the corresponding RNA-IP's using an anti- *hERG1a* antibody, followed by a bead-only control and H₂O control. The next group shows the corresponding RNA-IP's using the anti-Nav1.5 antibody, followed by a group of IgG controls. H₂O and beads lanes show absence of template contamination; control (+) represents signal amplified from purified plasmid template.

Figure S2: Specificity of the probes used in smFISH experiments. Representative images of smFISH for either *hERG1a* (top panel) or *SCN5A* (bottom panel) mRNAs performed in HEK293 cells transiently transfected with *hERG1a* or *SCN5A*. Only the cells expressing *hERG1a* or *SCN5A* showed a positive signal for smFISH revealing the specificity of the probes used in smFISH experiments.

a *hERG1a* mRNA

Model	Gauss		
Equation	$y=y_0 + (A/(w*\sqrt{\pi/2}))*\exp(-2*((x-xc)/w)^2)$		
Plot	Peak1	Peak2	Peak3
y0	0.49 ± 0.21	0.49 ± 0.21	0.49 ± 0.21
xc	117984.83 ± 2256.31	267703.93 ± 14735.19	527984.86 ± 76758.94
w	103169.32 ± 7458.84	222635.33 ± 40129.29	450302.22 ± 78120.74
A	$1.14E7 \pm 2.16E6$	$1.83E7 \pm 6.81E6$	$1.87E7 \pm 5.78E6$

b *SCN5A* mRNA

Model	Gauss		
Equation	$y=y_0 + (A/(w*\sqrt{\pi/2}))*\exp(-2*((x-xc)/w)^2)$		
Plot	Peak1	Peak2	Peak3
y0	0.71 ± 0.25	0.71 ± 0.25	0.71 ± 0.25
xc	129681.69 ± 1775.62	350694.45 ± 10021.78	746868.44 ± 133741.99
w	100467.91 ± 5381.31	332189.87 ± 38494	754612.46 ± 142927.70
A	$1.26E7 \pm 1.15E6$	$2.90E7 \pm 7.35E6$	$2.48E7 \pm 7.91E6$

c *GAPDH* mRNA

Model	Gauss		
Equation	$y=y_0 + (A/(w*\sqrt{\pi/2}))*\exp(-2*((x-xc)/w)^2)$		
Plot	Peak1	Peak2	Peak3
y0	0.35 ± 0.31	0.35 ± 0.31	0.35 ± 0.31
xc	354447.18 ± 11207.17	623535.81 ± 117199.66	$1.17E6 \pm 238744.98$
w	274182.43 ± 29650.81	444264.04 ± 165298.03	893413.86 ± 242256.38
A	$4.64E7 \pm 2.18E7$	$4.21E7 \pm 3.35E7$	$3.10E7 \pm 1.51E7$

Figure S3: Single mRNA intensity determination. The distribution of total fluorescence intensity of smFISH signals for *hERG1a* (2611 spots; **a**), *SCN5A* (2815 spots; **b**), and *GAPDH* (3507 spots; **c**). By fitting the histogram to the sum of Gaussian functions (red line), the typical intensity corresponding to a single mRNA molecule (vertical dashed line) was extracted.

Figure S4: Quantification of mRNA expression using two different methods. **a**, The distribution of total fluorescence intensity of smFISH signals for *hERG1a* (2892 spots) obtained using FISHQUANT software for analysis. By fitting the histogram to the sum of Gaussian functions (red line), the typical intensity corresponding to a single mRNA molecule (vertical dashed line) was extracted. **b**, Comparison of the number of mRNA molecules detected per cells for *hERG1a* using 2 different methods of analysis (Method 1: manual using ImageJ; Method 2: Semi-automatic using FISHQUANT).

Figure S5: Correlation of mRNA expressions. The number of mRNA molecules detected per cells in double smFISH experiments were plotted for *SCN5A* and *GAPDH* (28 cells, **a**), and *hERG1a* and *GAPDH* (13 cells, **b**). The Pearson's correlation coefficient (R^2) were calculated for each pairs of mRNAs.

Figure S6: hERG1a mRNA protein interaction. **a**, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to Immunofluorescence combined to smFISH protocol showing the colocalization (yellow arrows) of *hERG1a* mRNA (magenta) and hERG1a protein (green). **b**, Pie chart showing the percentage of *hERG1a* mRNA population interacting with hERG1a protein revealing that 16% of *hERG1a* mRNA were actively translated at the moment of fixation.

Figure S7: Co-knockdown of *hERG* and *SCN5A* mRNAs by *hERG* transcript-specific shRNA. a, Representative confocal images of smFISH for *hERG1a* and *SCN5A* transcripts in iPSC-CMs transfected with either a control or *hERG1b* shRNA. **b,** Histogram of the average number of transcripts detected per cell for *hERG1a* or *SCN5A* transcripts in presence of *hERG1b* shRNA compared to a scrambled shRNA (mean $\pm$ SE). **c,** Histogram of the mean number of *hERG1a* transcript colocalized with *SCN5A* transcript in cells silenced for *hERG1b* compared with control (mean $\pm$ SE).