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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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46

47 **ABSTRACT**

48

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 Pointes[1]. 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.

77

78   Cardiac I_{Kr} is critical for normal repolarization[2] 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 cotranslationally[7] and preferentially form heteromultimers[8].

81   Underlying heteromultimerization is the cotranslational association of *hERG1a* and *1b* mRNA

82   transcripts[9]. 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 repolarization[9].

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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)

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93   quantitatively reveals *hERG* and *SCN5A* transcript colocalization captured during protein

94   translation. Targeting *hERG* transcripts for shRNA degradation coordinately reduces *SCN5A*

95   transcript levels as well, along with native I_{Kr} and I_{Na} currents recorded from cardiomyocytes.

96   Thus, cotranslational association and regulation of transcripts is a novel mechanism

97   establishing and preserving a balance of I_{Kr} and I_{Na} in heart, where relative levels of these

98   currents are critical for normal action potential production and coordinated electrical activity.

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106 **RESULTS**

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

108 Using specific antibodies that target the N-terminus of hERG1a, we purified hERG1a protein

109 from induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and human ventricle

110 lysates and performed RT-PCR to identify associated transcripts ("RNA-IP"; Fig. 1a). As

111 previously reported[9], both *hERG1a* and *1b* transcripts co-immunoprecipitated with nascent

112 hERG 1a protein. Surprisingly, *SCN5A* transcripts encoding $Na_v1.5$ channels also copurified

113 with nascent hERG1a protein (Fig. 1b and Supplementary Fig. S1). The interaction appears

114 specific since neither ryanodine receptor RyR2 nor inward rectifier channel Kir2.1 (*KCNJ2*)

115 transcripts copurified as part of this complex. The counterpart experiment using anti-$Na_v1.5$

116 antibodies confirmed association of transcripts encoding hERG1a, hERG1b and $Na_v1.5$, but

117 not RyR2 (Fig. 1b). Bead-only controls showed no signal, indicating specific interactions of

118 antibodies with corresponding antigens. The association also occurred in HEK293 cells,

119 where additional controls showed that the antibodies used did not interact nonspecifically

120 with mRNA encoding the other ion channels or subunits (Supplementary Fig. S1).

121 Interestingly, when lysates independently expressing hERG1a and $Na_v1.5$ were mixed,

122 hERG1a antibodies copurified only *hERG1a* mRNA, and Nav1.5 antibodies copurified only

123 *SCN5A* mRNA, indicating that association of the two mRNAs requires their co-expression *in*

124 *situ*. In addition, the interaction between *hERG1a* and *SCN5A* does not require the presence

125 of *hERG1b* (Supplementary Fig. S1). This experiment demonstrates that transcripts

126 encoding hERG1a, hERG1b and $Na_v1.5$ physically interact within the cell and can be

127 copurified using antibodies targeting either nascent hERG1a or $Na_v1.5$ proteins. Their

128 association with either encoded protein implies the transcripts associate during protein

129 translation, or *cotranslationally*.

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131 **_hERG1a_ and _SCN5A_ transcript distribution**

132 To independently confirm *hERG1a* and *SCN5A* transcript association, we performed single-

133 molecule fluorescence *in situ* hybridization (smFISH) experiments in iPSC-CMs (Fig. 2a). We

5

134   used a combination of short DNA oligonucleotides (20 nucleotides), each labelled with a

135   single fluorophore, that bind in series on the target mRNA and collectively are detected as a

136   single fluorescent spot[11] (see Methods). Probes for *hERG1a* and *SCN5A* mRNAs were

137   designed with spectrally separable labels for simultaneous detection (Quasar 647 and 546

138   respectively; see Methods and Supplementary Fig. S2 for probe validation, and Table S1 for

139   list of probes)[12]. Punctate signal for each mRNA species appeared singly and in clusters (Fig.

140   1a, b). To evaluate mRNA copy number in each detected signal, we fitted the histogram of

141   the total fluorescence intensity of smFISH signals with the sum of Gaussian functions and

142   determined mean intensity of a single mRNA molecule for each species (Fig. 2b and

143   Supplementary Fig. S3). We found that approximately 25% of detected molecules exist

144   singly, whereas about 20% occupy clusters containing 6 or more transcripts (Fig. 2c). Both

145   transcripts were observed throughout the cytoplasm with higher density within 5-10 μm from

146   the nucleus (Fig. 2a, d), consistent with the expected distribution of perinuclear endoplasmic

147   reticulum where these mRNA molecules are translated into proteins. A *GAPDH* mRNA probe

148   set served as a positive control for smFISH experiments (Stellaris® validated control). In

149   contrast with signals observed for *hERG1a* and *SCN5A* transcripts, *GAPDH* transcript

150   clustered less, with 50% found as single molecules and <5% in clusters of 6 or more

151   transcripts (Fig. 2c). Moreover, *GAPDH* molecules distributed more homogeneously

152   throughout the cytoplasm with higher density in the range of 10 to 20 μm from the nucleus

153   (Fig. 2d)**.** We noted similar numbers of *hERG1a* and *SCN5A* transcripts per cell but fewer

154   than those for *GAPDH* (Fig. 2e). Thus, numbers and spatial distribution of *hERG1a* and

155   *SCN5A* transcripts can be simultaneously resolved. Further work will be required to elucidate

156   the significance or possible physiological role of differently sized mRNA clusters.

157

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

159   Although we observed a range in numbers of *hERG1a* and *SCN5A* mRNAs among iPSC-

160   CMs (Fig. 2e), regression analysis revealed clear correlation in their expression levels within

161   a given cell (Fig. 3 and Supplementary Table S3). Plotted against each other, *hERG1a* and

162   *SCN5A* mRNA numbers exhibited a coefficient of determination (R^2) of 0.57 (P=0.00001; 41

163   cells; Fig. 3a and b). In contrast, pairwise combinations of *hERG1a* and *RyR2*, *hERG1a* and

164   *GAPDH*, or *SCN5A* and *GAPDH* exhibited much lower linear correlation (R^2= 0.22, P=0.017;

165   R^2=0.18, P=0.15; and R^2=0.33, P=0.000134 respectively; n=26, 13, and 28 cells respectively;

166   Fig. 3c and d, Supplementary Fig. S5 a and b, and Supplementary Table S3). Spearman

167   coefficients revealed similar results as Pearson coefficients, where significant correlation is

168   observed only between *SCN5A* and *hERG1a* (Supplementary Table S3). These findings

169   indicate a roughly constant ratio of *hERG1a* and *SCN5A* mRNA copies.

170

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

172   To determine potential *hERG1a* and *SCN5A* transcript association using smFISH, we

173   measured proximity between the two signals using the centroid position, scored from

174   touching to 67% (1 pixel) overlap (Fig. 4a and b). To discern colocalization from random

175   overlap, we calculated the expected number of particles that could associate based on

176   chance only for the different association criteria. Two-tailed *t* tests with Bonferroni correction

177   revealed association between *hERG1a* and *SCN5A* transcripts significantly greater than that

178   expected by chance (see Methods; P values summarized in Supplementary Table S2; Fig.

179   4b). Approximately 25% of each transcript population was associated with the other (Fig. 4c).

180   To test specificity of interaction between *hERG1a* and *SCN5A* transcripts, smFISH and

181   pairwise comparisons were also performed with *RyR2* and *GAPDH* transcripts, which

182   revealed no significant association (Fig. 4d and e; Supplementary Table S2). These results

183   show that association of *hERG* and *SCN5A* transcripts demonstrated in lysates can also be

184   visualized in iPSC-CMs *in situ*, and provide strong evidence for the existence of a discrete

185   mRNA complex comprising *hERG1a* and *SCN5A* transcripts.

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189 **Discrete *hERG1a* and *SCN5A* cotranslational complexes**

190 To further explore whether colocalized mRNAs were part of a translational complex, we

191 combined smFISH with immunofluorescence using hERG1a antibodies. We observed close

192 association between *hERG1a* and *SCN5A* mRNAs and hERG1a protein significantly greater

193 than that expected by chance (Fig. 5a and b and Supplementary Fig. S6a and b).

194 Interestingly, among the 16% of actively translated *hERG1a* mRNAs (i.e. those associated

195 with hERG1a protein), 46% were also associated with *SCN5A* mRNAs (Fig. 5c), indicating a

196 3-fold enrichment of their association in translational complexes. Analysis of the distribution

197 of colocalized molecules revealed that 70% are located close to the nucleus (within 10μm,

198 Fig. 5d).

199

200 We monitored association of hERG1a protein and transcript in the presence of puromycin,

201 which releases translating ribosomes from mRNAs[13] (Fig. 6a). We observed no change due

202 to puromycin in the total number of respective mRNAs detected per cell (Fig. 6b). As

203 expected, puromycin reduced association between *hERG1a* mRNA and hERG1a protein

204 (antibody) and the S6 ribosomal protein (Fig. 6c). In addition, triple colocalization of *hERG1a*

205 and *SCN5A* transcripts and either hERG1a protein or the ribosomal subunit S6 was robustly

206 reduced (Fig 6d). These findings further support the conclusion that *hERG1a* and *SCN5A*

207 associate cotranslationally.

208

209 ***hERG1a* and *SCN5A* mRNAs are coregulated**

210 We previously demonstrated that targeted knockdown of either *hERG1a* or *1b* transcripts by

211 specific short hairpin RNA (shRNA) caused a reduction of both transcripts not attributable to

212 off-target effects in iPSC-CMs or in HEK293 cells[9]. To determine whether *hERG* and *SCN5A*

213 transcripts are similarly subject to this co-knockdown effect, we evaluated expression levels

214 by performing RT-qPCR experiments in iPSC-CM. We found that *hERG1a, hERG1b* and

215 *SCN5A* expression levels were all reduced by about 50% upon *hERG1a* silencing compared

216 to the effects of a scrambled shRNA (Fig. 7a, orange bars). *RYR2* transcript levels were

8

217   unaffected. We observed similar results using the specific hERG1b shRNA (Fig. 7a, blue

218   bars). Expressed independently in HEK293 cells, only *hERG1a* mRNA was affected by the

219   1a shRNA, and only *hERG1b* was affected by the 1b shRNA (Fig. 7b). *SCN5A* was

220   unaffected by either shRNA, indicating that the knockdown in iPSC-CMs was not due to off-

221   target effects and levels of associated *hERG1a* and *SCN5A* are quantitatively coregulated.

222   Similar results of approximately 40% co-knockdown of discrete *hERG1a* and *SCN5A* mRNA

223   particles were obtained using smFISH (Supplementary Fig. S7). Even more than the total

224   population of mRNA, the number of colocalized particles is decreased by approximately

225   55%, indicating that physically associated transcripts are subjected to co-knockdown (Fig.

226   S7c). Together these results indicate a coordinated and quantitative regulation of mRNAs

227   encoding a complement of ion channels.

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

230   To assess functional consequences of transcript coregulation, we recorded effects of

231   *hERG1b* silencing on native currents in iPSC-CMs. Fig. 7c shows the repolarizing current I_{Kr}

232   in iPSC-CMs transfected with either hERG1b or scrambled shRNA. Steady state and peak

233   tail I_{Kr} were decreased in *hERG1b*-silenced cells compared to cells transfected with

234   scrambled shRNA (Fig. 7d). I_{Kr} reduction was the result of a decrease in G_{max} upon hERG1b-

235   specific silencing with no modifications in the voltage dependence of activation (Fig. 7e and

236   Supplementary Table S4). These results are in accordance to our previous studies reporting

237   a reduction in I_{Kr} density upon *hERG1b*-specific silencing, and indicate that transcripts

238   targeted by shRNA are those undergoing translation[9,10]. To determine whether *hERG1b*

239   silencing also affects translationally active *SCN5A*, we measured peak I_{Na} density in iPSC-

240   CMs and detected significant reduction of about 60% when *hERG1b* was silenced, compared

241   to control cells (Fig. 7f, g and h). Peak G_{max} was decreased but no alterations in voltage

242   dependence of activation or inactivation were detected (Fig. 7h and Supplementary Tables

243   S4 and S5). Late I_{Na}, measured as the current integral from 50 to 800 ms from the beginning

244   of the pulse[14], was similarly reduced in magnitude (Fig. 7i, j and k). This analysis indicates

245   that coregulation via co-knockdown results in quantitatively similar alteration of $I_{Na,late}$ and I_{Kr},

246   which operate together to regulate repolarization[15]. I_{to}, which does not regulate action

247   potential duration in larger mammals[16], is unaffected by *hERG1b* silencing (Fig. 8a, b, c and

248   d), suggesting the coregulation of I_{Na} and I_{Kr} reflects their coherent participation in

249   repolarization.

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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*

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281   mRNA levels squares with previous reports of fixed channel transcript ratios associated with

282   certain identified crustacean neurons[23,24]. Cotranslating mRNAs in a stoichiometric manner

283   could buffer noise associated with transcription[25] and render a stable balance of channel

284   protein underlying control of membrane potential.

285

286   These studies raise questions of the mechanism by which transcripts associate. Although

287   hERG1a and hERG1b N-termini interact during translation[7], association of transcripts does

288   not rely on this interaction: alternate transcripts encoding the proteins interact even when

289   translation of one of the proteins is prevented[9]. In principle, transcripts could associate via

290   complementary base pairing or by tertiary structural interactions as ligand and receptor.

291   Alternatively, they could be linked by one or more RNA binding proteins (RBPs). Because the

292   association and coregulation observed in native heart can be reproduced in HEK293 cells,

293   the same or similar mechanisms are at work in the two systems. More work will be required

294   to discern among possible mechanisms, and to determine the time course with respect to

295   transcription, nuclear export and cytosolic localization of interacting transcripts.

296

297   A mechanism involving RBPs is appealing because it comports with the idea of the "RNA

298   regulon," a term describing a complex of transcripts bound by one or more RBPs[26,27]. RBPs

299   in the yeast Puf family bind large collections of mRNAs to control their localization, stability,

300   translation and decay[28,29]. In mammalian systems, the Nova protein serves to coordinate

301   expression of mRNAs encoding splicing proteins important in synaptic function[30].

302   Presumably in both cases these proteins interact in multiple regulons (complexes) serving

303   different or related roles. Mata and colleagues isolated individual mRNA species in yeast and

304   showed they associate with other mRNAs encoding functionally related (but nonhomologous)

305   proteins, along with mRNA encoding the RBP itself[31]. Moreover, these mRNAs encoded

306   proteins that formed stable macromolecular complexes[32]. Taking it one step further, Cosker

307   et al. showed that two mRNAs involved in cytoskeletal regulation bind the same RBP to form

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308   a single RNA granule[33], possibly analogous to the microtranslatome regulating key elements

309   of excitability in the heart reported here.

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311   A comprehensive analysis of the microtranslatome's components will require RNA-seq at a

312   level of multiplexing that ensures sufficient statistical power in the face of potentially reduced

313   complexity of the RNA-IP samples. These efforts will necessarily be followed by validation

314   through complementary approaches such as RNAi and smFISH to confirm their identity

315   within the microtranslatome.

316

317   One of the more curious findings of our study is the coordinate knockdown of different

318   mRNAs in the complex by shRNAs targeted to only one of the mRNA species. The

319   mechanism by which multiple mRNA species may be simultaneously regulated is not clear.

320   shRNAs silence gene expression by producing an antisense (guide) strand that directs the

321   RNA-induced silencing complex (RISC) to cleave, or suppress translation of, the target

322   mRNA[34,35]. Since hERG shRNA has no off-target effect on *SCN5A* mRNA expressed

323   heterologously in HEK293 cells, we assume there is insufficient complementarity for a direct

324   action. Perhaps by proximity to RISC, translation of the nontargeted mRNA is also disrupted,

325   but to our knowledge no current evidence is available to support this idea. A transcriptional

326   feedback mechanism seems unlikely given that co-knockdown can occur with plasmids

327   transiently expressed from engineered promoters and not integrated into the genome of

328   HEK293 cells. It is also important to note that it is unknown whether *SCN5A* is the only

329   sodium channel transcript coregulated by *hERG* knockdown. In principle, transcripts

330   encoding other sodium channels implicated in late I_{Na}, such as Nav1.8[36,37], could also be

331   affected, as could transcripts encoding auxiliary subunits associated with Nav1.5[38].

332

333   Whether disrupting the integrity of these complexes gives rise to some of the many

334   arrhythmias not attributable to mutations in ion channel genes *per se* remains to be

335   determined. Although the coregulation of inward I_{Na} and outward I_{Kr} shown in this study may

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336   suggest a compensatory mechanism, in a previous study we showed that selective

337   knockdown of *hERG1b* prolongs action potential duration and enhances variability, both

338   cellular markers of proarrhythmia[10]. Perhaps in the absence of co-regulation the effects

339   would be more deleterious. Jalife and colleagues have introduced the concept of the

340   "channelosome," a macromolecular protein complex mediating a physiological action.

341   Interestingly, Nav1.5 and Kir2.1, which regulates resting and diastolic membrane potential,

342   exhibit compensatory changes when the levels of either are genetically manipulated[39]. In this

343   case, the effect seems to be on stability of the nontargeted channel proteins, which form a

344   complex together with SAP97, and not on mRNA levels[40]. We do not yet know whether the

345   complex of transcripts we have studied encodes a similarly stable macromolecular complex,

346   or perhaps ensures appropriate ratios of channels distributed independently at the

347   membrane. Based on current evidence, we propose that the microtranslatome of associated

348   transcripts is a novel mechanism governing the quantitative expression of multiple ion

349   channel types and thus the balance of excitability in the cardiomyocyte.

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356   **METHODS**

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358   **Cell culture, plasmids and transfection**

359   HEK293 cells were cultured under standard conditions (37°C, 5% CO_2) in DMEM medium

360   (Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco). iPSC-CM (iCell®, Cellular

361   Dynamics International) were plated and cultured following manufacturer's instructions.

362   ShRNA sequences specific for hERG1a 5'-GCGCAGCGGCTTGCTCAACTCCACCTCGG-3'

363   and its control 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3' were provided by Origene

364   into a pGFP-V-RS vector. shRNA specific for hERG1b 5'-CCACAACCACCCTGGCTTCAT-3'

365   and its respective control were purchased from Sigma-Aldrich. For heterologous expression,

366   hERG1a (NM_000238) and hERG1b (NM_172057) sequences were cloned into pcDNA3.1.

367   Transient transfections were performed using 2.5 µl/ml Lipofectamin™ 2000 (Thermofisher)

368   with 2 µg/ml plasmid. Cells were collected for further analysis 48h after transfection. When

369   needed, a second transfection was performed 24h after the first one with either hERG1a or

370   hERG1b shRNA and the corresponding scrambled shRNA as a control. Cells were then

371   collected for experiments 48h after last transfection.

372

373   **Antibodies**

374   Rabbit anti-hERG1a (#12889 from Cell Signaling, 1:100), rabbit anti-hERG1b (#ALX-215-051

375   from Enzo, 1:100), rabbit anti-pan hERG (#ALX-215-049 from Enzo, 1:3000), rabbit anti

376     $Na_V1.5$ (#ASC-005 from Alomone or #D9J7S from Cell signaling, 1:500), were used for

377   immunofluorescence, western blot or RNA-IP experiments. Alexa 647 goat anti-rabbit, Alexa

378   488 goat anti-rabbit or Alexa 488 donkey anti-mouse were employed for indirect

379   immunofluorescence or immunoblotting experiments (Thermofisher; 1:1000).

380

381   **RNA isolation and semi-quantitative real-time PCR**

382   RNA isolation and purification were achieved using TriZol reagent (Life Technologies) and

383   RNeasy Mini Kit (Qiagen). RT-qPCR experiments were performed using a TaqMan Gene

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384   Expression Assay (Life Technologies) and mRNA expression levels were calculated using

385   the $2^{-\Delta\Delta Ct}$ cycle threshold method. All data were normalized to mRNA level of *β-actin*

386   housekeeping genes. Because iPSC-CMs are subject to inherent biological variability, we

387   used a standardization procedure to normalize the independent biological replicates as

388   previously described[41]. Briefly, a log transformation of the normalized relative expression

389   gene level was performed, followed by mean centering and autoscaling of the data set.

390   Results are expressed as average and 95% confidence intervals. Primers were purchased

391   from Invitrogen (*hERG1a*: Hs00165120_m1; *hERG1b*: Hs04234675_m1; *SCN5A*:

392   Hs00165693_m1; *RYR2*: Hs00892883_m1; and *β-actin*: Hs01060665_g1).

393

394   **Immunofluorescence**

395   For immunofluorescence studies, iPSC-CMs were grown on gelatin-coated coverslips, rinsed

396   in PBS three times and fixed in 4% paraformaldehyde for 10 minutes at room temperature.

397   Following fixation, cells were incubated 1h at room temperature with a solution containing

398   0.5% triton 100X for permeabilization and 1% bovine serum albumin along with 10% serums

399   (secondary antibodies species) diluted in PBS to saturate samples and limit nonspecific

400   binding. Cells were then processed for indirect immunofluorescence using a combination of

401   primary and secondary antibodies (see antibodies section above). Cells were washed three

402   times with PBS, incubated with DAPI to counterstain nuclei and mounted with Vectafield

403   mounting medium.

404

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

406   FISH was performed using Stellaris® probe sets, which comprised up to 48 oligonucleotides

407   designed to selectively bind in series the targeted transcripts. Probes were designed using

408   the StellarisTM Probe Designer by LGC Biosearch Technologies with the following

409   parameters: masking level: 5, oligo length: 20 nucleotides, and minimum spacing length: 2

410   nucleotides. Oligonucleotides were labeled with TAMRA or Quasar® 670 dyes for detection of

411   *SCN5A* and *hERG* respectively. 48 oligonucleotides were designed for *SCN5A*, *RyR2* and

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412   *GAPDH* and 35 for the specific N-terminal sequence of *hERG1a*. Sequences for all probes

413   are provided in Supplementary Table 1. FISH was performed on iPSC-CMs according the

414   manufacturer's protocol. Briefly, fixation was performed by adding paraformaldehyde to a

415   final concentration of 4% (32% solution, EM grade; Electron Microscopy Science) followed by

416   a hybridization step for at least 4h at 37°C in a buffer containing a final concentration of 125

417   nM probes and 10% formamide (Stellaris hybridization buffer). Cells were washed for 30 min

418   (Stellaris washing buffer A) before incubation for 30 min at 37°C with DAPI to counterstain

419   the nuclei. A final washing step was performed (Stellaris washing buffer B) and coverglasses

420   were mounted onto the slide with Vectashield mounting medium.

421   Digital images were acquired using a 63X objective on a Leica DMi8 AFC Inverted wide-field

422   fluorescence microscope. Z-sections were acquired at 200 nm intervals. Image pixel size:

423   XY, 106.3 nm. Image post-treatments were performed using ImageJ software (NIH). Briefly,

424   a maximum projection was performed before background subtraction and images were

425   filtered using a Gaussian blur filter to improve the signal/noise ratio and facilitate spot

426   detection. Spot detection and colocalization was performed using the plugin ComDet on

427   ImageJ[42,43].

428   FISHQUANT was used as a second method for spot detection and gave similar values.

429   Briefly, background was substracted using a Laplacian of Gaussian (LoG) and spots were fit

430   to a three-dimensional (3D) Gaussian to determine the coordinates of the mRNA molecules.

431   Intensity and width of the 3D Gaussian were thresholded to exclude non-specific signal[11,12].

432   To evaluate the number of mRNA molecules, the total fluorescence intensity of smFISH

433   signals was fitted with the sum of Gaussian functions (see equation below) to determine the

434   mean intensity of a single mRNA.

435

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

436

17

437

438 **Statistical analysis of smFISH and IF**

439 For the purpose of our statistical calculations, we assumed that the protein and mRNA

440 signals were circular. The following formulas were used to calculate the expected number of

441 mRNAs (E_m) that would interact based on chance alone for each association criteria:

442

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

443

444 where N_{m1} is the total number of mRNA in one channel, N_{m2} is the total number of mRNA in

445 the second channel, r is the average radius of mRNA spots (in nm), I is the intersection

446 between particles (nm^2), and A is the total area of the region analyzed (in nm^2). As the

447 distance between particles is increased, the number of expected associated mRNAs will

448 increase since more mRNAs will be considered associated. We used criteria with different

449 stringency in the first set of experiments (from 1 pixel to 4 pixels distance between spots) and

450 considered the 2 pixels distance between spots physiologically relevant for triple association

451 analysis and co-knockdown experiments.

452 To test the significance of triple associations between hERG1a mRNA, SCN5A mRNA and

453 hERG1a protein, the following formula was used:

454

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

455

456 where N_p is the total number of proteins, E_m is the expected number of mRNA that would

457 interact based on chance alone as calculated above. For each association criteria, the

458 intersection between particles was calculated using the following equation:

459

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

18

460

461

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 determine using a F test.

470

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 determine using two-tailed

probability test.

476

477

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.

19

484

485 **Electrophysiological measurements**

486 Patch clamp under whole-cell configuration was used to record all ionic currents. I_{Kr} and

487 $I_{Na,late}$ were recorded at physiological temperatures (37°C), while I_{Na} was recorded at room

488 temperature (22°C) using an Axon 200B amplifier and Clampex Software (Molecular

489 Devices). Glass pipettes with a resistance of 2.5 – 5 MΩ measured with physiological

490 solutions (below) were pulled using an automatic P-97 Micropipette Puller system (Sutter

491 Instruments).

492

493 To record steady state and tail I_{Kr}, cells were continuously perfused with an external solution

494 containing (in mM): NaCl 150, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1, Glucose 15, HEPES 15, Na-

495 pyruvate 1, and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with an internal

496 solution containing (in mM): NaCl 5, KCl 150, CaCl$_2$ 2, EGTA 5, HEPES 10, Mg-ATP 5, and

497 the pH was adjusted to 7.3 with NaOH. The voltage protocol for I_{Kr} was completed at

498 physiological temperature (37°C) and determined as an E-4031 (2µM) sensitive current.

499 Cells were recorded using a holding potential of -50 mV, followed by a pulse at -40 mV to

500 inactivate sodium channels, then 3-second depolarizing steps (from -50 to +30 mV in 10 mV

501 increments) to activate hERG channels and finally to -40 mV for 6 seconds. Steady-state I_{Kr}

502 was measured as the 5 ms average current at the end of the depolarizing steps. Tail currents

503 were measured following the return to -40 mV.

504 To record I_{Na}, cells were perfused with an external solution containing (in mM): NaCl 50,

505 Tetraethylammonium (TEA) methanesulfonate 90, CaCl$_2$ 2, MgCl$_2$ 1, Glucose 10, HEPES 10,

506 Na-pyruvate 1, Nifedipine 10 µM, and pH adjusted to 7.4 with TEA-OH. Micropipettes were

507 filled with an internal solution containing (in mM): NaCl 10, CaCl$_2$ 2, CsCl 135, EGTA 5,

508 HEPES 10, Mg-ATP 5, and pH was adjusted to 7.3 with CsOH.

509 I_{Na} activation was investigated by applying pulses between -140 and +20 mV in 10 mV

510 increments from a holding potential of -120 mV. To measure inactivation of sodium channels,

20

511   conditioning pulses from -140 to +20 mV in 10 mV increments were applied from a holding

512   potential of -120 mV following by a test pulse to -20 mV.

513   To record $I_{Na,late}$, cells were perfused with an external solution containing (in mM): 140, CsCl

514   5.4, $CaCl_2$ 1.8, $MgCl_2$ 2, HEPES 5, Nifedipine 10 µM, and pH was adjusted to 7.3 with NaOH.

515   Pipette were filled with an internal solution containing (in mM): NaCl 5, CsCl 133, Mg-ATP

516   2, TEA 20, EGTA 10, HEPES 5, and pH was adjusted to 7.33 with CsOH. $I_{Na,late}$ was

517   measured by applying an 800 ms single pulse to -30 mV from a holding potential of -120 mV.

518   The mean current was measured at the last 200 ms of the pulse. An external solution

519   containing 30 µM TTX was perfused after the first pulse to determine if the current was due

520   to the activity of sodium channels.

521   To record I_{to}, cells were continuously perfused with an external solution containing (in mM):

522   NaCl 150, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Glucose 15, HEPES 15, Na-pyruvate 1, E4031 2,

523    $CdCl_2$ 0.5 and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with an internal

524   solution containing (in mM): NaCl 5, KCl 150, $CaCl_2$ 2, EGTA 5, HEPES 10, Mg-ATP 5, and

525   the pH was adjusted to 7.3 with NaOH.

526   Both activation (for I_{Kr}, Ito and I_{Na}) and inactivation (for I_{Na}) were fitted to Boltzmann equations

527   (Equations (1) and (2), respectively) and voltage dependence parameters were obtained.

528

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

529
530

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

531
532

533

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21

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542   **Author Contributions**

543   Experiments were conceived and designed by C.A.E, E.R.-P., F.L. and G.A.R. C.A.E. and

544   M.B.J. carried out single molecule FISH experiments. F.L. and J.J.K. conducted RNA-IP and

545   knock-down experiments. E.B.R-P. and D.K.J. performed electrophysiology experiments.

546   C.A.E. and G.A.R. wrote the manuscript and all authors provided critical feedback to the final

547   version.

548

549   **Data Availability**

550   The source data corresponding to Figures 1b, 2b, 2c, 2d, 2e, 3b, 3d, 4b, 4c, 4e, 5b, 5c, 5d,

551   6b, 6c, 6d, 7a, 7b, 7d, 7e, 7g, 7h, 7j, 7k, 8a, 8b, 8d and Supplementary Figures S1, S3a,

552   S3b, S3c, S4a, S4b, S5a, S5b, S6b, S7b, S7c. Raw files images and other data supporting

553   the findings of this study are available upon request.

554

555

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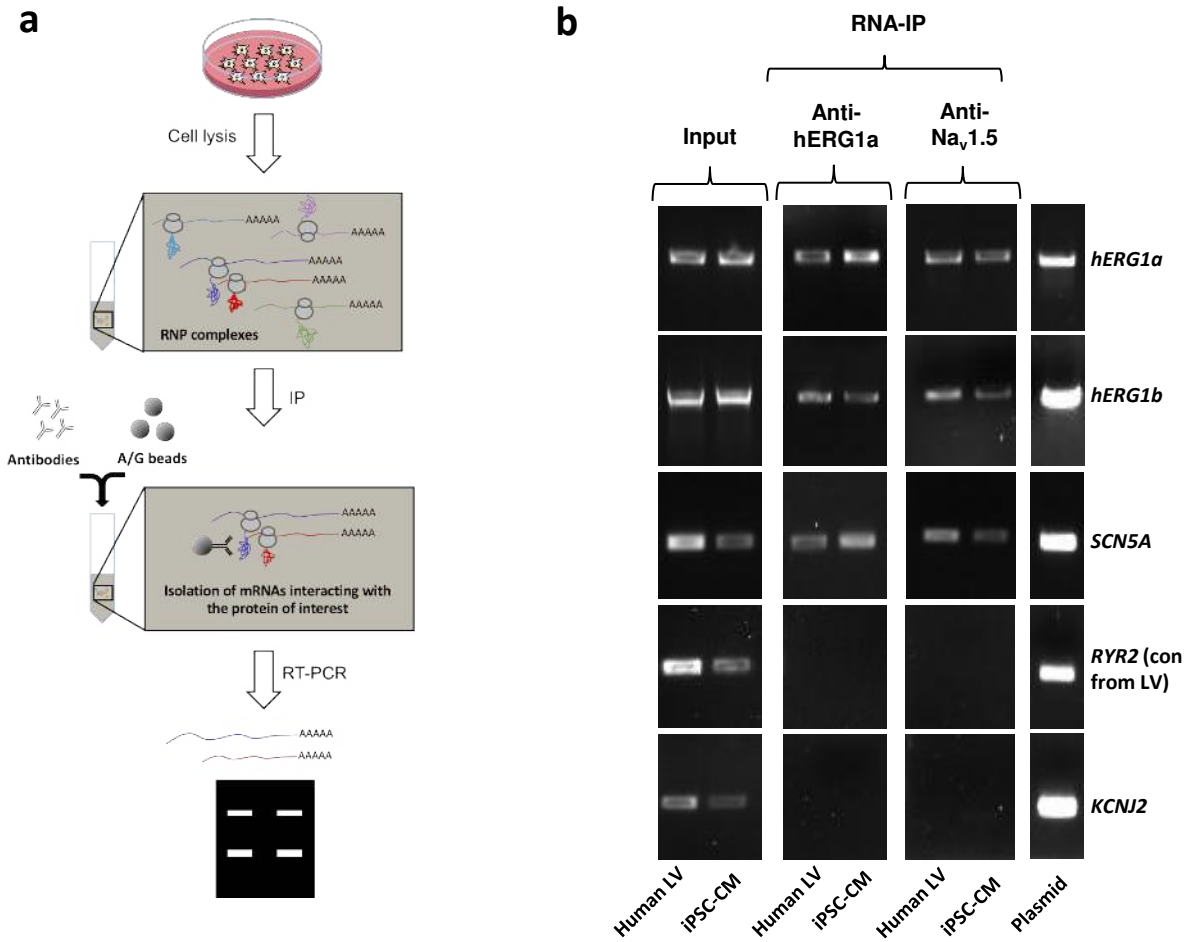
666

Fig. 1

a

b

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-Na$_v$1.5 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).

Fig. 2
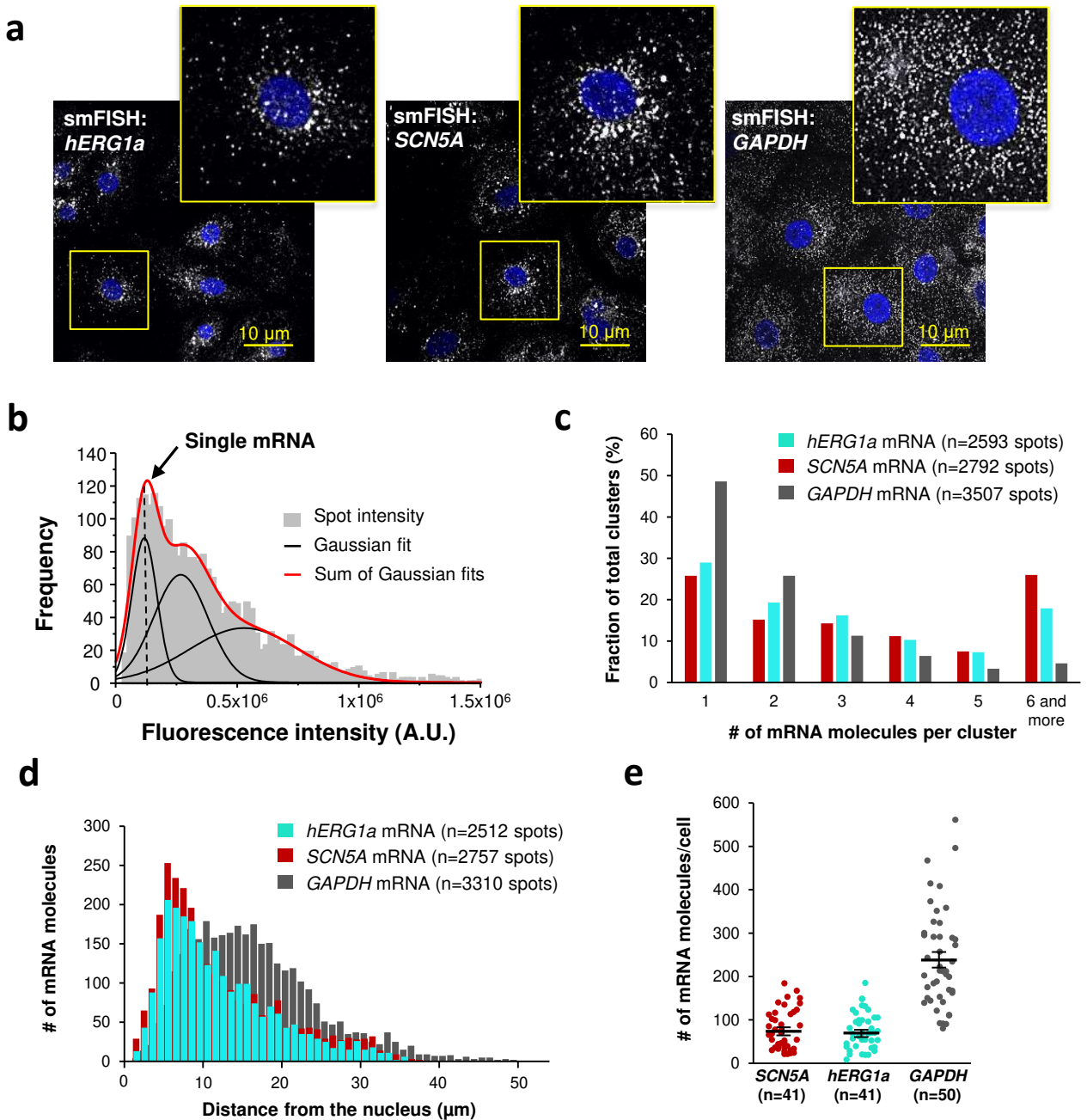
Figure 2: Quantitative description of single *hERG1a* and *SCN5A* transcripts and their distribution in iPSC-CMs. a, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to the smFISH protocol. **b,** By fitting the intensity histogram of smFISH signals (n=2611 spots) 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 ± SE).

Fig. 3
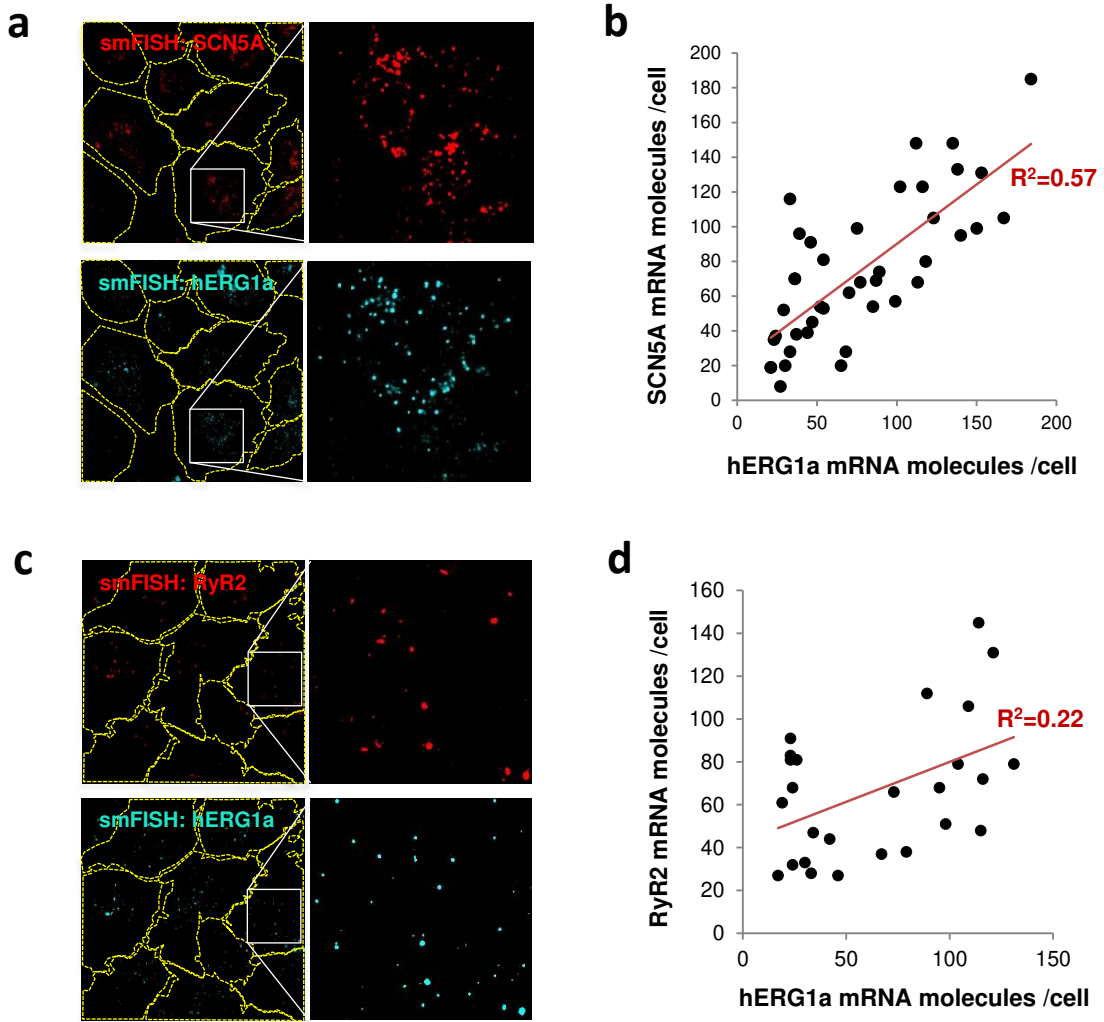
Figure 3: *hERG1a* and *SCN5A* transcript expression levels correlate. a, Representative confocal images and enlargements of double smFISH experiments for *SCN5A* (red) and *hERG1a* (cyan) mRNAs. **b,** The number of mRNA molecules detected per cell in double smFISH experiments were plotted for *SCN5A* 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).

Fig. 4
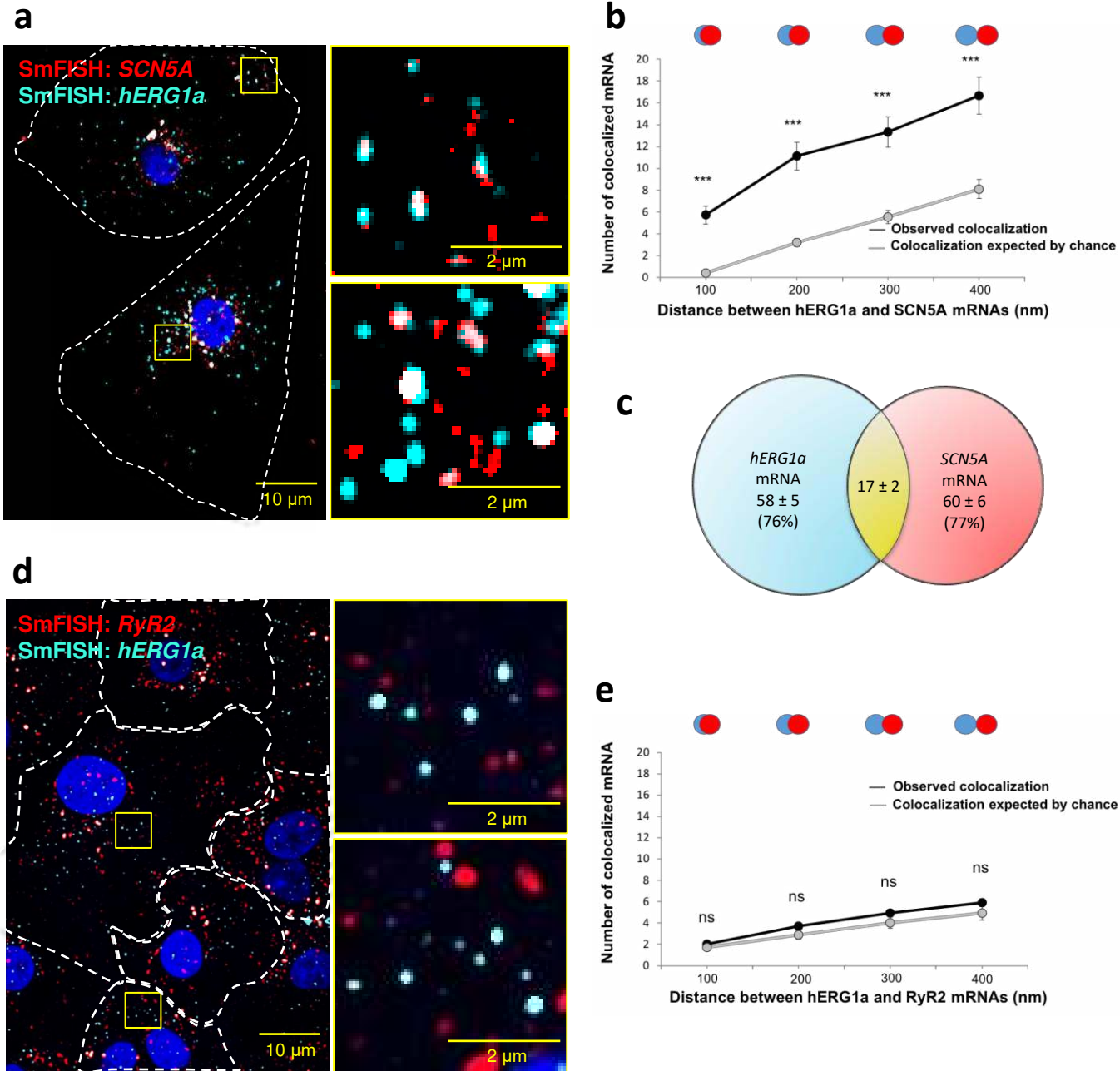
Figure 4: hERG1a and SCN5a transcript colocalization. a, Representative confocal images and enlargement (outlined in yellow) of iPSC-CMs subjected to smFISH showing the colocalization of *hERG1a* and *SCN5A* mRNAs. **b,** Comparison of the average number of associated *hERG1a* and *SCN5A* mRNAs particles observed vs. expected by chance using different overlap criter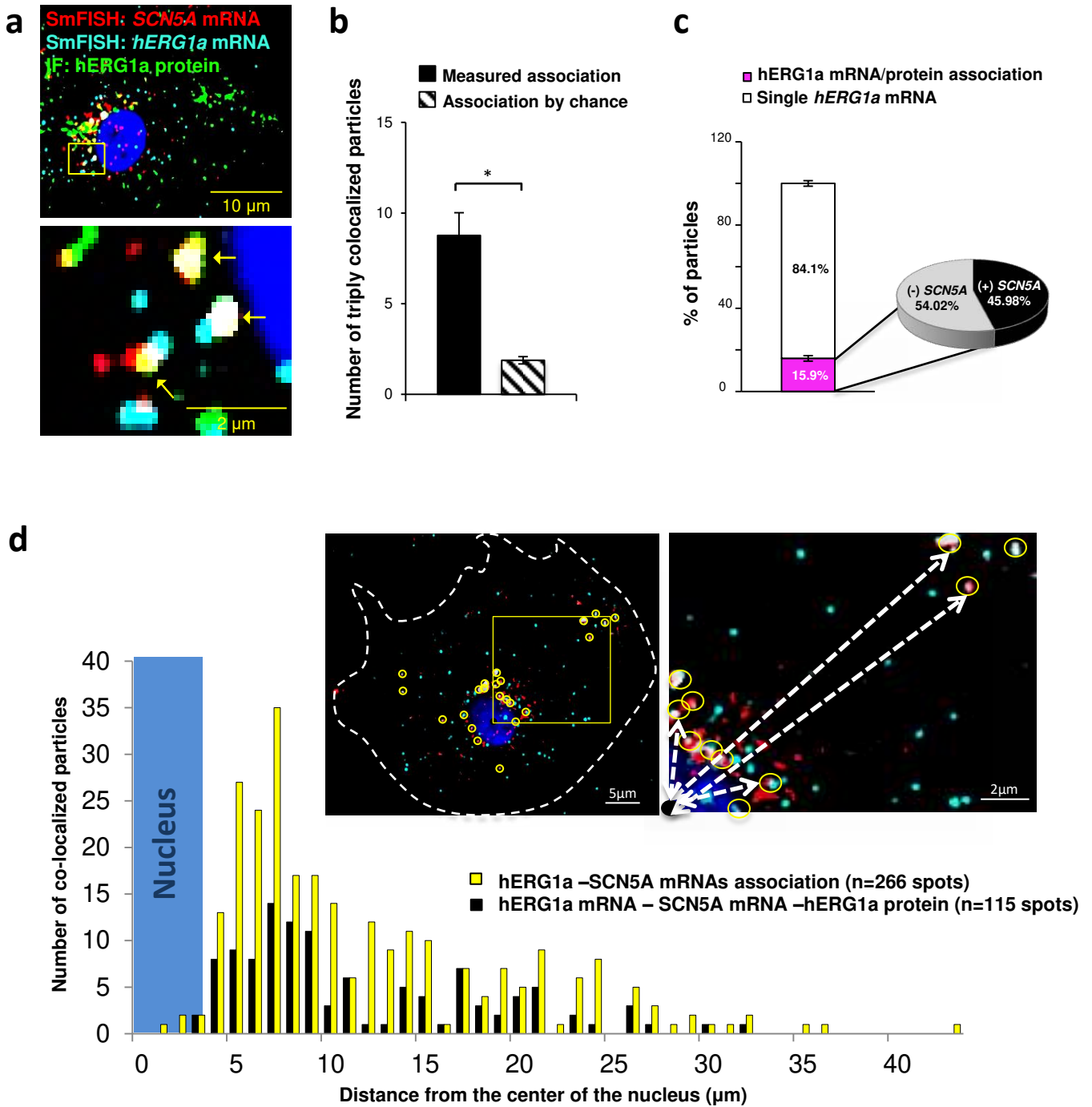ia illustrated (mean ± SE; n=41 cells; N=2). **c**, Diagram illustrating that the association of hERG1a and SCN5A mRNAs account for 24% and 23% of their total population respectively. **d,** Representative confocal images of smFISH for hERG1a and RyR2 transcripts. **e,** Comparison of the average number of associated *hERG1a* and *RyR2* mRNAs particles observed vs. expected by chance using different overlap criteria (mean ± SE; n=26 cells; N=2).

Fig. 5

a
SmFISH: *SCN5A* mRNA
SmFISH: *hERG1a* mRNA
IF: hERG1a protein
10 µm
2 µm

b
■ Measured association
▨ Association by chance
Number of triply colocalized particles
*

c
■ hERG1a mRNA/protein association
□ Single *hERG1a* mRNA
% of particles
84.1%
15.9%
(-) *SCN5A* 54.02%
(+) *SCN5A* 45.98%

d
Number of co-localized particles
Nucleus
5µm
2µm
□ hERG1a –SCN5A mRNAs association (n=266 spots)
■ hERG1a mRNA – SCN5A mRNA –hERG1a protein (n=115 spots)
Distance from the center of the nucleus (µm)

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 ± 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 ± 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).
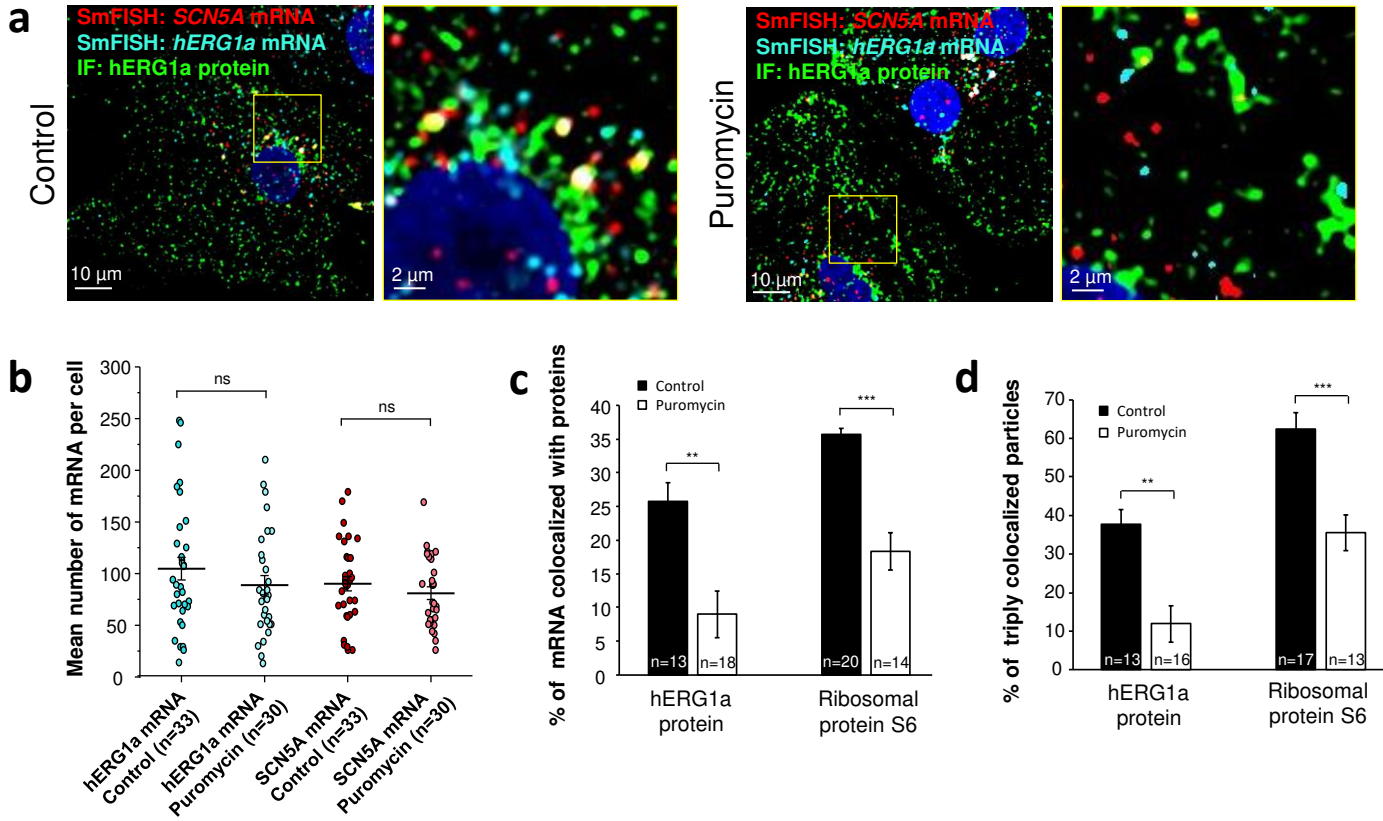
Fig. 6

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 µM 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 ± SE). **c,** Histogram showing the reduction of association between *hERG1a* mRNA and hERG1a protein after puromycin treatment compared to non-treated cells (mean ± 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 ± SE).

Fig. 7
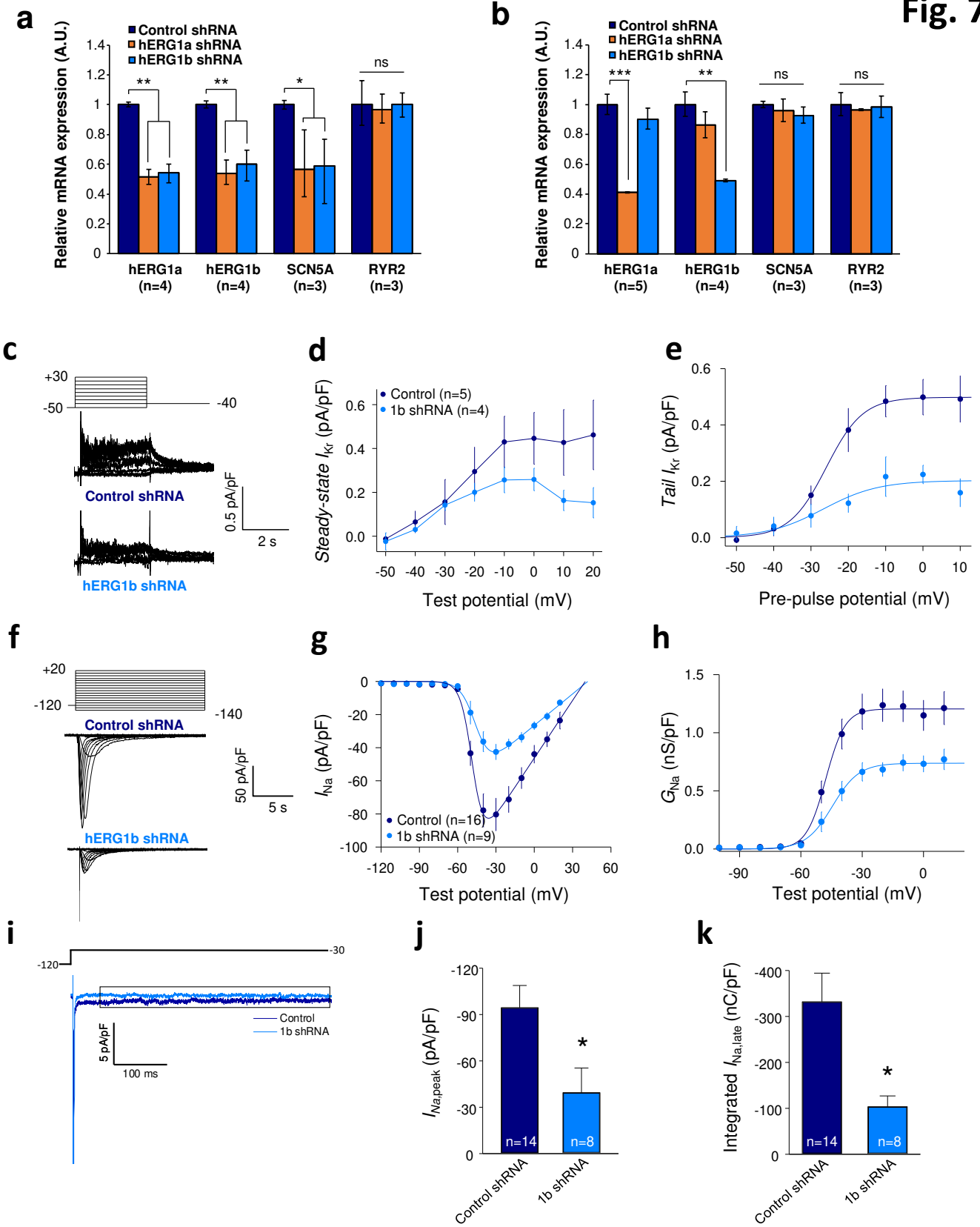
Figure 7: Co-knockdown of I_{Kr} and I_{Na} by hERG transcript-specific shRNA. a, Effects of *hERG1a* or *hERG1b* silencing on channel mRNA expression levels detected by RT-qPCR (mean ± 95% CI) in IPSC-CMs. A non-targeting shRNA (scrambled shRNA) is used as a control. **b,** Effects of specific *hERG1a* 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 ± SE). **e**, Effects of 1b shRNA on peak tail current vs. pre-pulse potential (mean ± 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 ± SE). **h**, Summary conductance (G)-voltage plot based on data from *g* (mean ± 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 ± 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 ± SE).
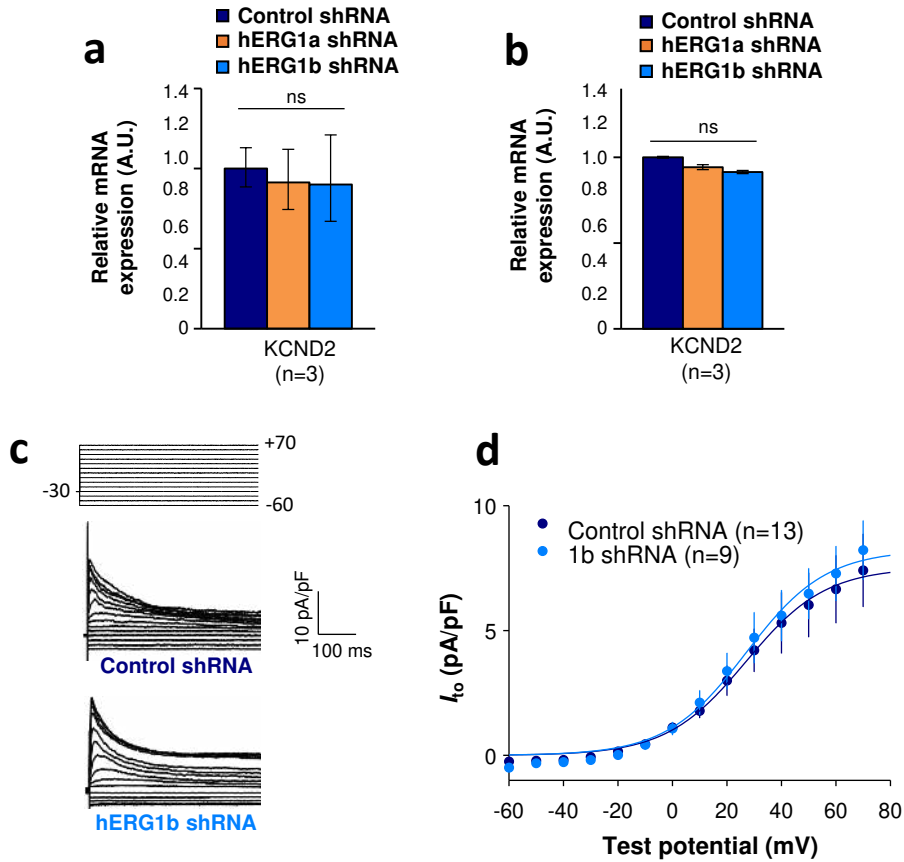
Fig. 8

a

■ Control shRNA
■ hERG1a shRNA
■ hERG1b shRNA

b

■ Control shRNA
■ hERG1a shRNA
■ hERG1b shRNA

c

-30 ___ +70

-60

10 pA/pF

100 ms

Control shRNA

hERG1b shRNA

d

● Control shRNA (n=13)
● 1b shRNA (n=9)

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 ± 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 ± SE).

Supplementary

hERG1a probes	SCN5A probes	RyR2 probes
caagactggactgcgggc	cccgaggtaataggaagttt	tccatttttccacatcaact
agcactaggcttcgggtg	tagagatctggcagcttttt	acatccaatttttccttgga
caggaaggtgttctgcgg	agtcttttgggtgctataga	tgtatagatcccattctcac
acttgcggatgatggtgt	gaagatggtcttgcctttat	catgctgggatctcgacaaa
aacttacggctctggccc	ggactgaggacatacaaggc	caaggcccttataaatcta
ccgagcgttggcgatgat	gtgtactcgacatacttggt	gaagtagccaatgagatcct
agatgacggcgcagttct	gaccagagactcaaaggtgt	tctgttctgtttgtcttcat
cagaagccgtcgttgcag	ccgaaggaaagtgaacgcgt	agtcgagggagccagaaaat
cgagtagccgcacagctc	aatcacactaaagtccagcc	acatccagaaccttgtgatt
gcagggtcgctgcatcac	attcagttgtgtatgccatg	tggcaaacacagagtgagca
cgtgcaggaagtcgcagg	tgcgtaaggctgagacattg	tcacagatgagatgctggtt
cagtgcctgcgcgatctg	ccaggcaaaggaatcgaagc	atggttcacaagacgtgtct
gatttccactttgcgctc	caggcggaagagtgcaagaa	gatgaaggccatcaaatcca
cccatctttccggtagaa	tcagtaaacgggtccatgac	ttggtgagcttacagtacga
ccaccagacataggaagc	tgtgttgagtacgatgcaca	aggttgtccattaattcgga
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aacatgatgacagcccca	gggcaatgatcttgaaggtc	acttgtcagctggtaattct
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gtggttggtgtcatgagc	ccgatgatcttgatgagtgt	cacggaagattcggaacctt
cttcaggcggaaggtctt	gaacacgatgatggctagca	gtgtgaacatcatggtgtgt
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ctttctcgggagcgcgtc	cactgctgagtaggatcatg	tttcctgggcataatctttg
catggcctcgatgtcgtc	ctaggtagatgtcctcgaag	ggctttgttcttctaagcaa
tgcgcagtgggtgcatgg	caagcagaaccttgatggtc	tacgtggacgtttgcatcaa
gtggagttgagcaagccg	atctccagcacgaagacata	agtcaaagcctgtgtcatac
cacgaggtcggagtccga	agtacttcttgaagccgtag	tggcaatgaatgtgagcagc
tgctaatggtgcggtagc	aatcgtgacagagctctcag	ggacttgtagcttgtgcaaa
ttgagggtgatttgggga	cgaggaggacgttcatgatg	gagagaggcatcacattctt
gcccttgaggtccacaaa	tgttcacgatggtgtagttc	ttcttgtgctcactcttgaa
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gatctcacggtcactggt	ccacgttgtcaaagttgact	gggacaatgtactcgttgtt
ctcctttatcttaggtgc	gctgcatacataatgtccat	gcttacaaaactgggggagg
	tcctctgtcatgaagatgtc	acacactgggctcaatcaac
	catggcattgtagtacttct	ttgatctgttcttgaggtgg
	aatatgaagccctggtactt	agacgccctctaattgttaa
	catcatggtcaccatattca	agtcactctcaactggtttt
	cagcttgacaatacactcgc	gggtcttcaatgacagactc
	ttggtgaagtagtagtggcg	ctccgaatgagcaatctcac
	tggagaggatgacaaccacg	atggacctaattctgatggc
	aagagcgtcggggagaagaa	tgaacctcaatcccatagac
	catcatgagggcaaagagca	ctgtgcaaaggtaccgattg
	acatgacgaggaagagcagc	agagaggagcacatcttgtt
	ccatgccaaagatggagtag	gaacagcccttagaaagtct
	cccacttgacataagcgaag	aaacttctatggaatcccgc
	taggagatcttggatgggtt	ttctgagtaagtgctgcatc

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.

	400 nm Touching	300 nm 20% overlap	200 nm 45% overlap	100 nm 67% overlap	
hERG1a/SCN5A (n=41 cells)	Expected by chance: 8.10 ± 0.87 Measured: 16.68 ± 1,70 P value: 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: 4.92 ± 0.66 Measured: 5.92 ± 0.36 P value: 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: 14.87 ± 2.65 Measured: 13.54 ± 1.68 P value: 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: 17.31 ± 1.71 Measured: 15.92 ± 1.64 P value: 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 perfomed 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.

		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 ρ	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 perfomed 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 adjust 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.

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$_{max\ peak\text{-}tail}$ (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.
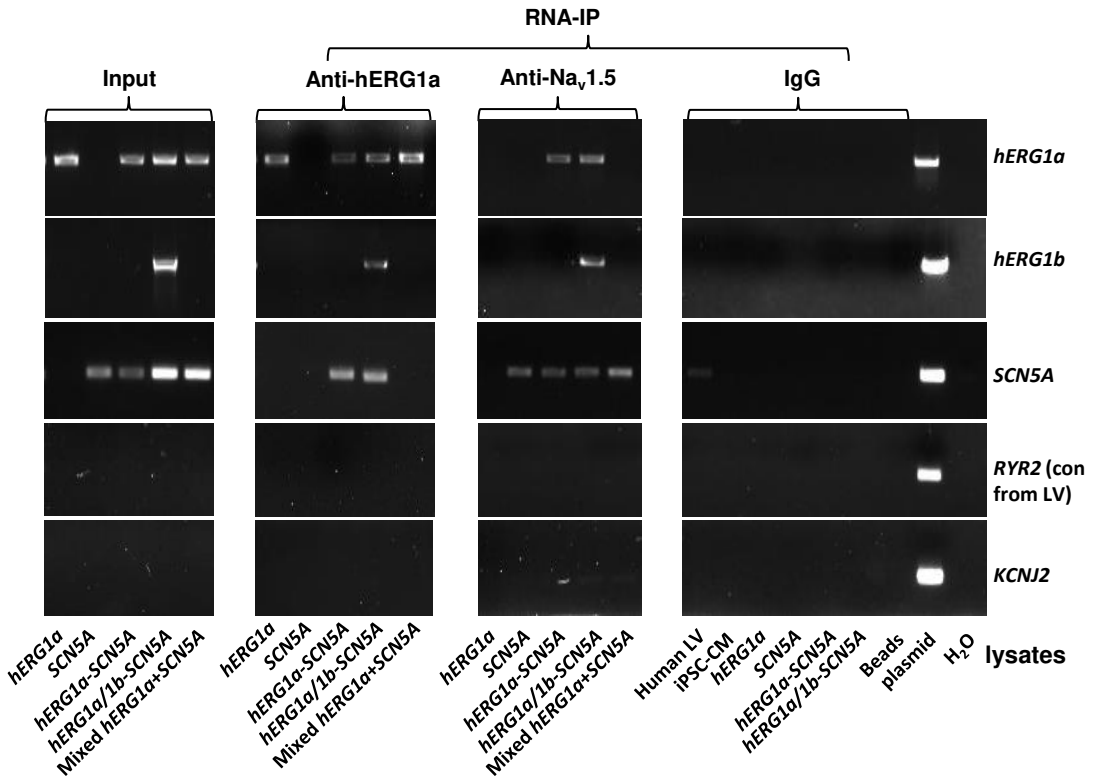
Figure S1: Complete RNA-IP from Figure 1. *Lanes 1-6,* RT-PCR products from input lysate of human left ventricle (LV), iPSC-CM, and HEK293 cells expressing: *hERG1a*; *SCN5A*; *hERG1a* plus *SCN5A*; and *hERG1a* plus *hERG1b* and *SCN5a*. *Lane 7* shows RT-PCR product from lysates independently expressing *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.
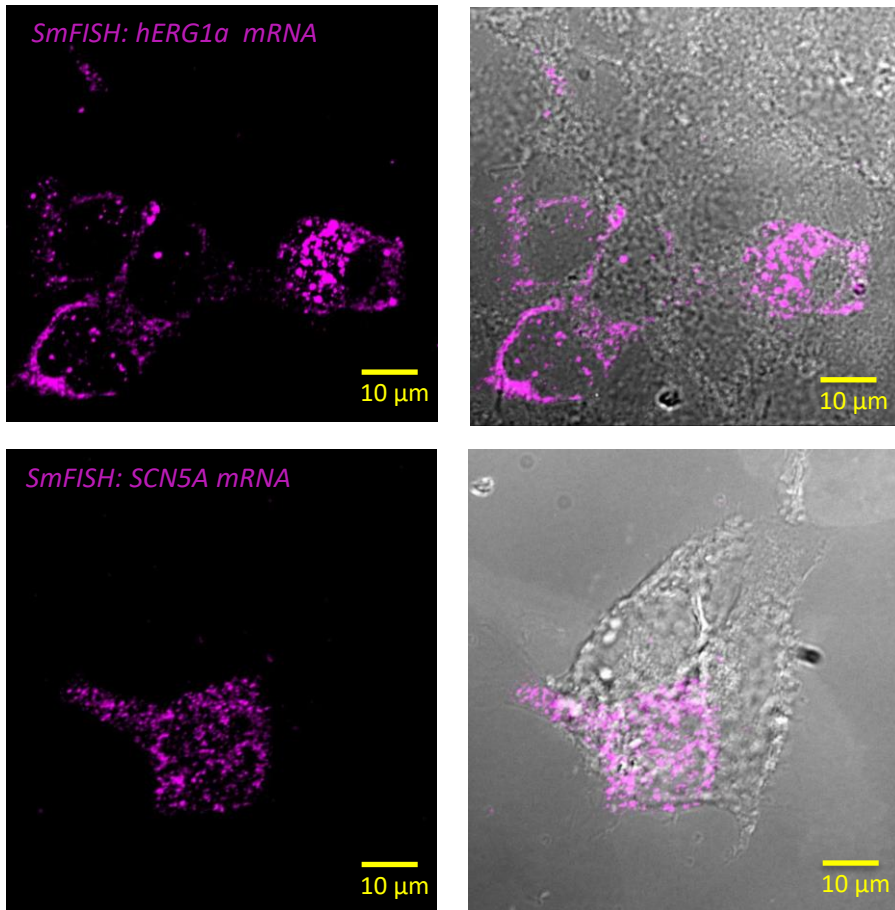
Figure S2: Specificity of the probes used in smFISH experiments. Representative images of smFISH for either hERG1a (top panel) or SCN5A (bottom panel) mRNAs performed in HEK293 cells transiently transfected with *hERG1a* or *SCN5A*. Only the cells expressing *hERG1a* or *SCN5A* showed a positive signal for smFISH revealing the specificity of the probes used in smFISH experiments.

a *hERG1a mRNA*
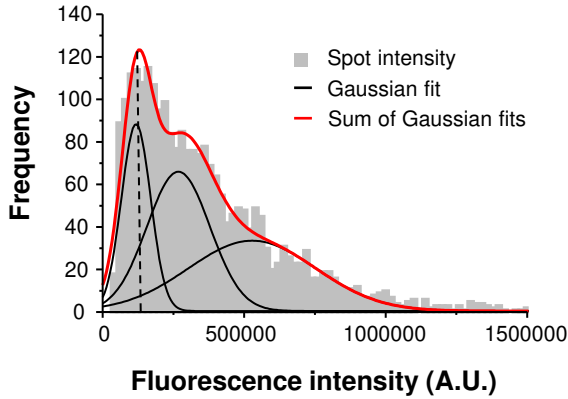
Model	Gauss		
Equation	y=y0 + (A/(w*sqrt(pi/2)))*exp(-2*((x-xc)/w)^2)		
Plot	Peak1	Peak2	Peak3
y0	0.49 ± 0.21	0.49 ± 0.21	0.49 ± 0.21
xc	117984.83 ± 2256.31	267703.93 ± 14735.19	527984.86 ± 76758.94
w	103169.32 ± 7458.84	222635.33 ± 40129.29	450302.22 ± 78120.74
A	1.14E7 ± 2.16E6	1.83E7 ± 6.81E6	1.87E7 ± 5.78E6

b *SCN5A mRNA*
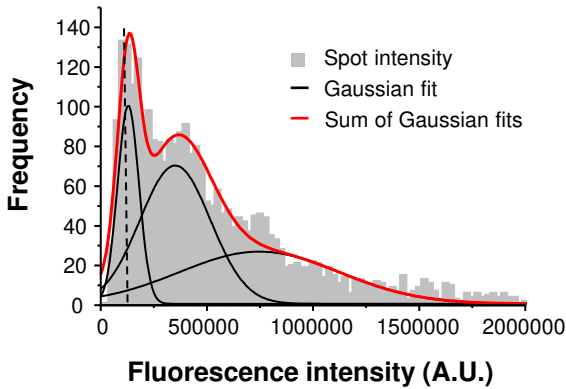
Model	Gauss		
Equation	y=y0 + (A/(w*sqrt(pi/2)))*exp(-2*((x-xc)/w)^2)		
Plot	Peak1	Peak2	Peak3
y0	0.71 ± 0.25	0.71 ± 0.25	0.71 ± 0.25
xc	129681.69 ± 1775.62	350694.45 ± 10021.78	746868.44 ± 133741.99
w	100467.91 ± 5381.31	332189.87 ± 38494	754612.46 ± 142927.70
A	1.26E7 ± 1.15E6	2.90E7 ± 7.35E6	2.48E7 ± 7.91E6

c *GAPDH mRNA*
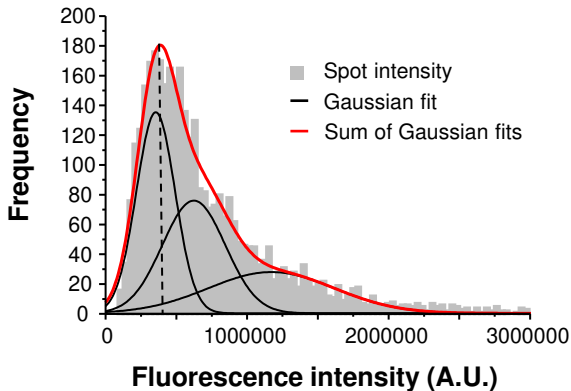
Model	Gauss		
Equation	y=y0 + (A/(w*sqrt(pi/2)))*exp(-2*((x-xc)/w)^2)		
Plot	Peak1	Peak2	Peak3
y0	0.35 ± 0.31	0.35 ± 0.31	0.35 ± 0.31
xc	354447.18 ± 11207.17	623535.81 ± 117199.66	1.17E6 ± 238744.98
w	274182.43 ± 29650.81	444264.04 ± 165298.03	893413.86 ± 242256.38
A	4.64E7 ± 2.18E7	4.21E7 ± 3.35E7	3.10E7 ± 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.
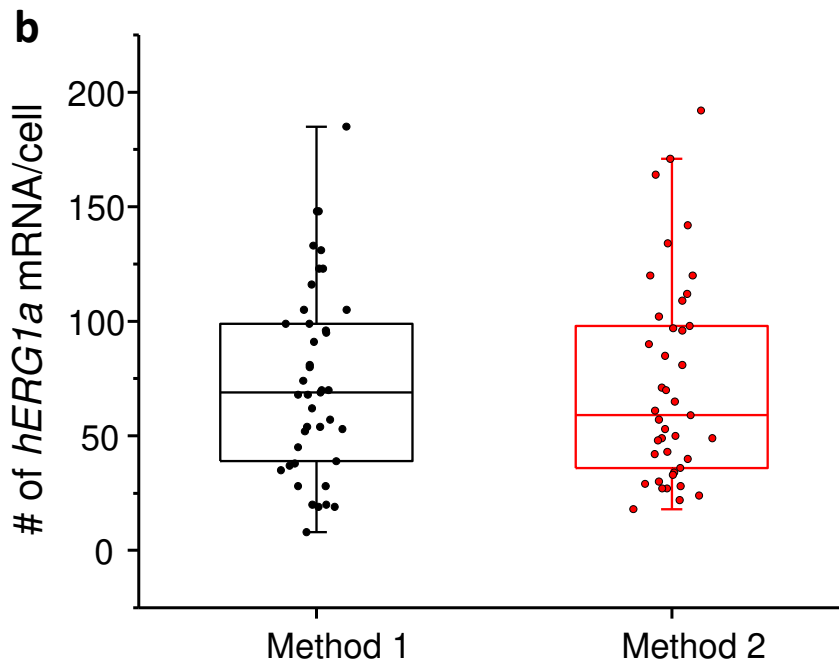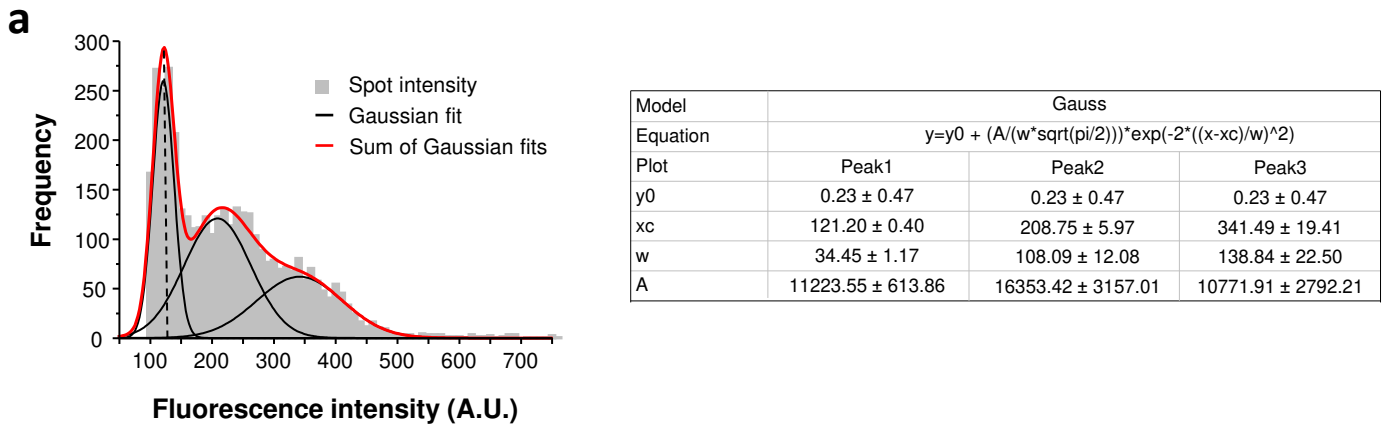
a

Model	Gauss		
Equation	y=y0 + (A/(w*sqrt(pi/2)))*exp(-2*((x-xc)/w)^2)		
Plot	Peak1	Peak2	Peak3
y0	0.23 ± 0.47	0.23 ± 0.47	0.23 ± 0.47
xc	121.20 ± 0.40	208.75 ± 5.97	341.49 ± 19.41
w	34.45 ± 1.17	108.09 ± 12.08	138.84 ± 22.50
A	11223.55 ± 613.86	16353.42 ± 3157.01	10771.91 ± 2792.21

b

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).
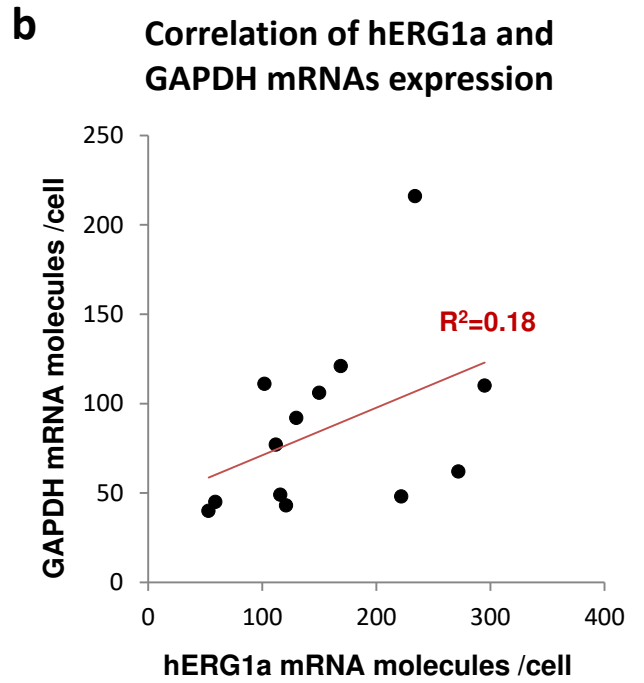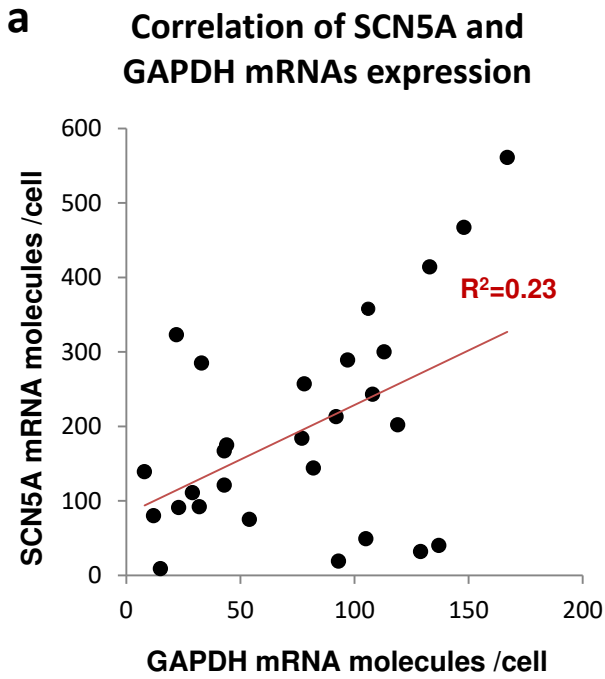
a

Correlation of SCN5A and GAPDH mRNAs expression

$R^2=0.23$

b

Correlation of hERG1a and GAPDH mRNAs expression

$R^2=0.18$

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 *hERG1*a and *GAPDH* (13 cells, **b**). The Pearson's correlation coefficient (R^2) were calculated for each pairs of mRNAs.
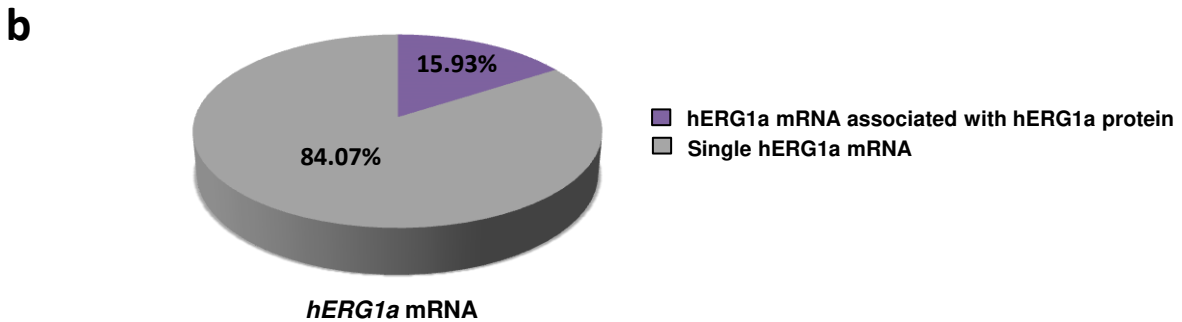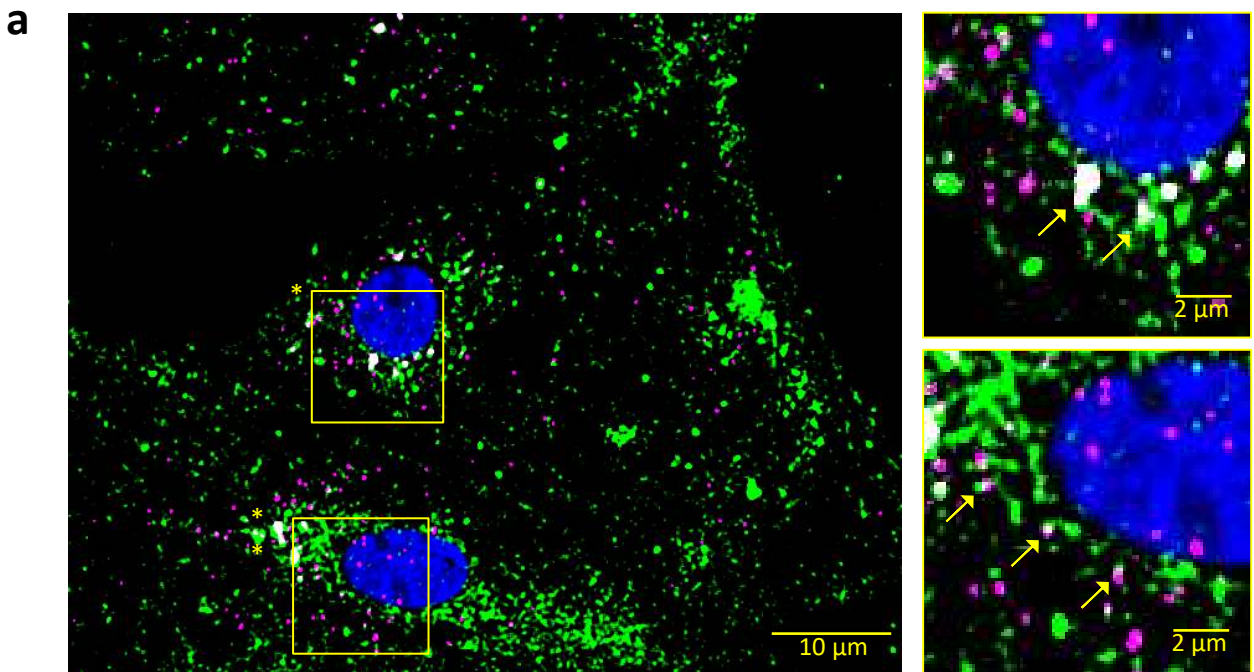
a

b

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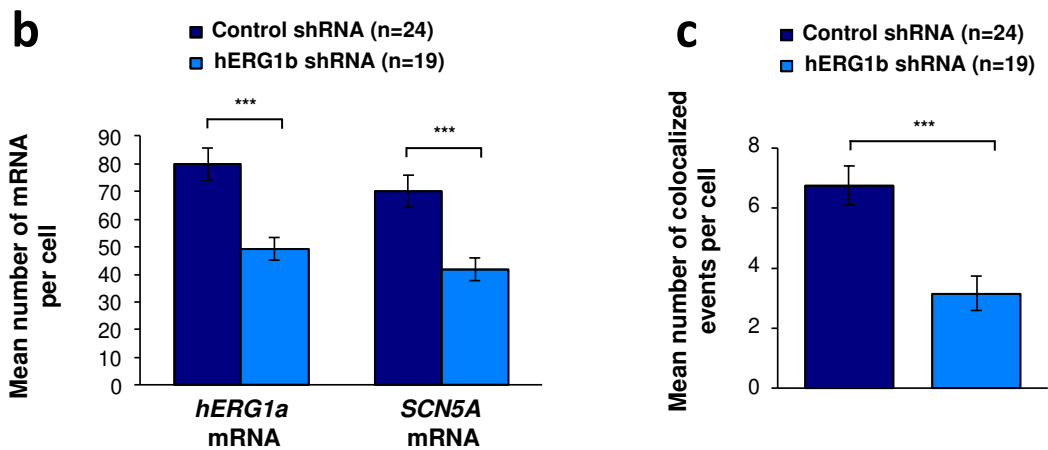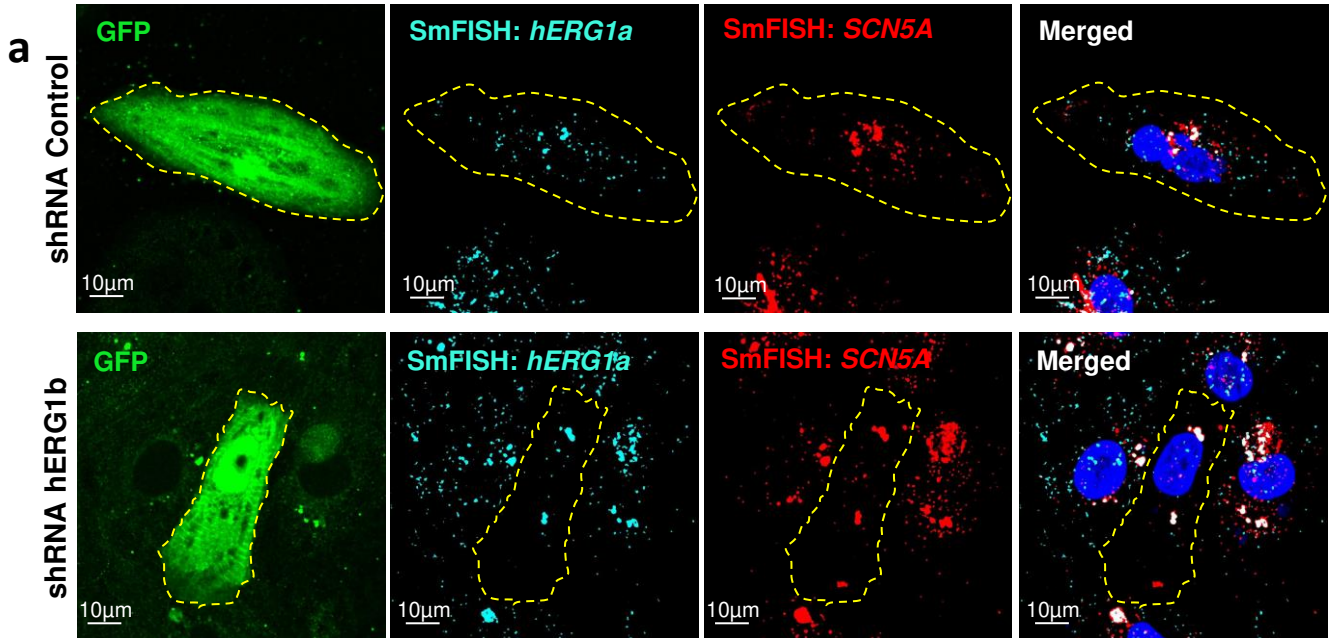
Figure S7: Co-knockdown of *hERG* and *SCN5A* mRNAs by hERG transcript-specific shRNA. a, Representative confocal images of smFISH for *hERG1a* and *SCN5A* transcripts in iPSC-CMs transfected with either a control or hERG1b shRNA. **b,** Histogram of the average number of transcripts detected per cell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