

1 **Tyrosine kinase inhibitors induce mitochondrial dysfunction**
2 **during cardiomyocyte differentiation through alteration of**
3 **GATA4-mediated networks**

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Highlights

- Early-stage exposure to TKIs induced cardiotoxicity and mitochondrial dysfunction
- GATA4 transcriptional activity is inhibited by TKIs
- Network analysis reveals interactions between GATA4 and mitochondrial genes
- GATA4-overexpression rescues cardiomyocytes and mitochondria from TKI exposure

91 **SUMMARY**

92 Maternal drug exposure during pregnancy increases the risks of developmental
93 cardiotoxicity, leading to congenital heart defects (CHDs). In this study, we used
94 human stem cells as an *in-vitro* system to interrogate the mechanisms underlying
95 drug-induced toxicity during cardiomyocyte differentiation, including anticancer
96 tyrosine kinase inhibitor (TKI) drugs (imatinib, sunitinib, and vandetanib). H1-
97 ESCs were treated with these drugs at sublethal levels during cardiomyocyte
98 differentiation. We found that early exposure to TKIs during differentiation
99 induced obvious toxic effects in differentiated cardiomyocytes, including
100 disarranged sarcomere structure, interrupted Ca²⁺-handling, and impaired
101 mitochondrial function. As sunitinib exposure showed the most significant
102 developmental cardiotoxicity of all TKIs, we further examine its effect with in-vivo
103 experiments. Maternal sunitinib exposure caused fetal death, bioaccumulation,
104 and histopathologic changes in the neonatal mice. Integrative analysis of both
105 transcriptomic and chromatin accessibility landscapes revealed that TKI-
106 exposure altered GATA4-mediated regulatory network, which included key
107 mitochondrial genes. Overexpression of GATA4 with CRISPR-activation restored
108 morphologies, contraction, and mitochondria function in cardiomyocytes upon
109 TKI exposure early during differentiation. Altogether, our study identified a novel
110 crosstalk mechanism between GATA4 activity and mitochondrial function during
111 cardiomyocyte differentiation, and revealed potential therapeutic approaches for
112 reducing TKI-induced developmental cardiotoxicity for human health.

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122 INTRODUCTION

123 Drug-induced cardiotoxicity is one of the major causes of cardiac diseases, and
124 the underlying mechanisms include mitochondrial dysfunction, altered expression
125 of cardiac genes, and oxidative stress ¹. Drug exposure during cardiac
126 development increases the risks of developmental cardiotoxicity and congenital
127 heart defect (CHD), which is the most common birth defect affecting nearly 1% of
128 newborns every year ^{2,3}. Drug-induced developmental cardiotoxicity exerts
129 significant impacts on the quality of life and increases health care costs in the
130 U.S. However, unlike CHDs caused by chromosomal abnormalities or gene
131 mutations, the mechanisms underlying abnormalities and defects in cardiac
132 development from non-inherited factors (such as maternal exposure to chemicals)
133 is poorly understood, leading to challenges in prediction and prevention of drug-
134 induced developmental toxicity ⁴.

135 Human stem cells provide a great opportunity for mechanistic and predictive
136 developmental toxicology studies. Cellular differentiation from stem cells can be
137 used to recapitulate embryonic developmental process ^{5,6}. Chemical perturbation
138 during stem cell differentiation allows us to understand the impact of drug toxicity
139 on development as well as the underlying molecular mechanisms. We have
140 previously applied human stem cells towards a better understanding of the
141 transcription regulation driving 13-*cis*-retinoic-acid-induced disruption in
142 mesoderm formation ⁷.

143 In this study, we performed chemical perturbation with various drugs during
144 cardiac differentiation from H1 human embryonic stem cells (H1-ESCs). These
145 drugs were classified as pregnancy category C, D and X by the FDA's previous
146 version of the Pregnancy and Lactation Labeling Rule (PLLR), meaning that they
147 have been determined to pose a high risk of birth defects in humans. The drugs
148 selected for this study have been reported to exert high risk of CHD, including
149 tyrosine kinase inhibitors (TKIs) for cancer chemotherapy (imatinib, sunitinib and
150 vandetanib; category D) ⁸⁻¹⁰, immunosuppressants (tacrolimus [category C] and
151 mycophenolate [category D]) ^{11,12}, and thalidomide (known as teratogen
152 [category X]) ^{13,14}. Unlike category X drugs which are not allowed for use in

153 pregnant women, category C and D drugs may be used during pregnancy if
154 clinical benefits outweigh their risks. For instance, anti-cancer drugs (e.g., TKIs)
155 are capable of crossing the placental barrier and posing high risks of CHDs^{9,15};
156 although cases of pregnant women with cancer are uncommon, with incidences
157 of approximately 1-2/1000^{16,17}. Single cases of related CHDs from maternal
158 exposure to cancer drugs have been documented in clinical reports^{8,10,18,19}.

159 Transcription regulatory mechanisms during cardiomyocyte differentiation
160 upon drug exposure were explored in the present study, by integrative network
161 analysis of genome-wide transcriptomics and chromatin accessibility. We
162 discovered a novel a crosstalk mechanism between transcription factor GATA4
163 and mitochondrial function and biogenesis upon TKI exposure. Results from this
164 study will help us to fill knowledge gaps in our understanding of the mechanisms
165 of cardiac and metabolic dysfunctions due to drug exposure, and will benefit the
166 predictive toxicology for prevention of drug-induced toxicity for human health in
167 the future.

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171 **RESULTS**

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173 **Developmental exposure to sublethal level of TKIs induces disarrangement** 174 **of sarcomere and alteration in Ca²⁺-handling of cardiomyocytes**

175 We utilized a well-established protocol²⁰ for cardiomyocyte (CM) differentiation,
176 which consistently generates high-purity beating CMs derived from human stem
177 cells. In order to optimize the viability of differentiated CMs for the following
178 function analysis, we first determined the no-observed-adverse-effect-levels
179 (NOAELs) of these drugs for CM differentiation by dose-responsive experiments
180 (Supplemental Figure 1A). The NOAEL for TKI drugs (imatinib, sunitinib and
181 vandetanib) was 250 nM, and this is lower than their concentrations in blood
182 based on the clinical data^{15,21-25}. In addition, The NOAELs for tacrolimus and
183 mycophenolate were 50 nM, and the NOAEL for thalidomide was 100 nM. H1-

184 hESCs were then exposed to each drug at the corresponding NOAEL throughout
185 the CM differentiation, and 0.1% of DMSO was used as a vehicle control
186 (Supplemental Figure 1A).

187 Drug exposures were conducted using two experimental strategies, including:
188 Exposure I, a brief early exposure design, in which the TKI drugs were added
189 from day 0 to day 6 (hereafter referred to as the cardiac progenitor stage ⁷);
190 Exposure II, and a chronic exposure strategy, in which cells were exposed to TKI
191 drugs throughout the entire differentiation process until CMs were collected for
192 analyses (Figure1A). No significant differences in efficiency of CM differentiation
193 were observed between control and drug-treated groups (Figure1B and
194 Supplemental Figure 1B). However, in Exposure II, imatinib and sunitinib
195 (250nM) induced obvious disarrangements of myofilaments of differentiated
196 CMs, whereas 250nM of vandetanib induced only modest adverse effects on
197 sarcomere structure (Figure1C). To evaluate the impacts of drug-exposure on
198 differentiated CMs, spontaneous Ca^{2+} transients were analyzed in the
199 differentiated CMs after day 25. We observed TKI-induced abnormal Ca^{2+} -
200 handling in both Exposure I and II, including decreases in beating rates and
201 amplitude, increases in time to peak, TD90, TD50, and impaired calcium
202 recycling (*i.e.*, increased decay tau) (Figure 1D-1G). Both Exposure I and II of
203 TKIs exhibited similar effects on Ca^{2+} handling of differentiated CMs.

204 To determine whether these toxic effects were specific to anti-cancer TKIs,
205 we also evaluated other drugs associated with maternal-fetal toxicity. We found
206 that NOEALs of tacrolimus, mycophenolic acid, and thalidomide caused
207 dysfunction in Ca^{2+} -handling (Supplemental Figure 2A) in differentiated CMs;
208 while no significant morphological change was observed. These results
209 demonstrate that, at NOAELs, developmental exposure to TKI drugs caused
210 more severe effects during CM differentiation than non-TKI drugs used in this
211 study.

212

213 **Transcriptomic analysis revealed correlation between the transcriptional**
214 **regulatory network and mitochondrial function**

215 In order to elucidate the changes in gene expression during CM differentiation
216 due to developmental drug exposure, we performed genome-wide transcriptomic
217 analysis of differentiated CMs (day 20) using RNA-sequencing (RNA-seq)
218 experiments. Twelve modules (*i.e.*, networks) were identified by weighted
219 correlation network analysis (WGCNA) (Figure 2A), and the full lists of genes and
220 enriched GO terms are shown in Supplemental Tables 1 and 2. Clustering
221 analysis of the transcriptomic profiles revealed that TKI drug-treated CMs
222 exhibited high concordance between Exposure I and II (except Exposure I of
223 imatinib) (Figure 2B). We observed that gene expression within a module 1
224 exhibited down-regulation patterns in TKI-treated groups compared to other
225 groups (*i.e.*, immunosuppressant drugs, thalidomide, and 0.1% DMSO), and the
226 representative enriched-GO terms of this module are related to “heart
227 development” and “mitochondrial respiratory chain complex I assembly” (Figure
228 2C). Genes known to regulate cardiac development were assigned to this
229 module, such as *GATA4*, *MEF2A*, *TBX20*, and *HAND2*. In addition, genes
230 involved in oxidative phosphorylation (OXPHOS) (such as *NDUFV3* and
231 *NDUFA12*) and glycolysis (such as *HK1* and *ALDOA*) were also assigned to this
232 module (Figure 2D). Whereas exposure to TKIs generally led to down-regulated
233 gene expressions in this module, exposure to immunosuppressant and
234 thalidomide drugs had the opposite effect (Figure 2E), suggesting that TKIs
235 induced adverse effects on both cardiac functions and metabolisms *via*
236 dysregulation of transcription factors (TFs) (such as *GATA4*) and metabolic
237 genes, which exhibited some correlations during CM differentiation.

238 Differentiated CMs mainly use OXPHOS to support their large ATP demands
239 ²⁶⁻²⁸; therefore, we examined mitochondrial respiratory activity in TKI-treated
240 CMs. We found that chronic exposure to TKIs decreased the levels of both basal
241 respiration and maximal respiration in differentiated CMs (Figure 2F). The ATP
242 production was also decreased in TKI-treated cells, and sunitinib-treated cells
243 exhibited the lowest ATP level compared to other groups (Figure 2G). By
244 transcriptomic profiling of the genes involved in Ca²⁺-handling, ion channels, and
245 β -adrenergic signaling, we showed that TKIs caused down-regulation of *RYR2*,

246 *CAMK2D*, and *MYH7*, which are closely related to the regulation of CM
247 contraction (Supplemental Figure 2B). These results suggested that
248 developmental exposure to TKIs dysregulated expression of genes involved in
249 cardiac differentiation, contractile functions, and metabolism; impaired
250 mitochondrial respiration and ATP productions; and ultimately weakened
251 contraction of CMs.

252

253 **Motifs enrichment analysis reveals features of transcription-factor** 254 **occupancy in TKI-treated cardiomyocytes**

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256 Many of the genes disrupted by TKI exposure encode key transcription factors
257 important for CM differentiation (Figure 2A). We then leveraged ATAC-seq to
258 identify chromatin accessibility that correspond to altered TF occupancy^{29,30}.
259 Specifically, we measured TF-motif enrichments within differential ATAC-seq
260 peaks between control and TKI-treatment groups, and identified several motifs
261 that are significantly enriched in regions where chromatin accessibility was lost in
262 the TKI-treated groups compared to control (Figure 3A). These enriched motifs
263 correspond to several TF families, that are known to play important roles in CM
264 differentiation (such as GATAs, TEADs, and MEF2 family members) (Figures 3B
265 and Supplemental Figure 3A). The control group exhibited higher densities of
266 motifs for GATA4 and TEAD1/3 (Figure 3C) and stronger ATAC-seq signals
267 (accessibilities) around the summit of the motifs for GATA4 and TEAD1/3,
268 compared to that of TKI-treated cells (Figures 3D-3F; Supplemental Figure 3B).
269 The sunitinib-treated cells showed the most significant loss of chromatin
270 accessibility surrounding the TF motifs, suggesting that sunitinib exposure may
271 have the strongest adverse effects on DNA-binding of these TFs during CM
272 differentiation. Altogether, these results suggest that developmental exposure to
273 TKIs leads to significant changes in the binding patterns of critical developmental
274 TFs, leading to transcriptional dysregulation that may ultimately underlie the
275 toxicity of drug exposure during CM differentiation.

276

277 **Integrative genome-wide transcriptomic and open-chromatin analyses**
278 **revealed GATA4 as an important regulator for TKI exposure**

279
280 We further integrated both chromatin landscapes and gene expression data to
281 explore transcriptional regulatory networks (Figure 3G), using paired expression
282 and chromatin accessibility (PECA) model, which can elucidate the effect of TFs
283 bound to the activated context-specific *cis*-regulatory elements on the
284 transcription of target genes^{31,32}. We found that genes within the module 1 from
285 the WGCNA, which are involved in mitochondrial biogenesis and function, are
286 predicted to be regulated by transcription factors known to control heart
287 development, such as GATA4, MEF2A/C and TEAD1/3 (Figure 3G). These
288 genes include peroxisome proliferator-activated receptor alpha (*PPARA*) and
289 peroxisome proliferator-activated receptor gamma coactivator 1-alpha
290 (*PPARGC1A*, encoding PGC-1 α , which is a key regulator for mitochondrial
291 biogenesis)³³⁻³⁵, complex I (*i.e.*, *NDUF* gene family), and cardiac development
292 (such as *TBX5*)³⁶ (Figure 3G). Importantly, Expression levels of these TFs were
293 down-regulated in differentiated CMs upon exposure to TKIs (Figure 3H). In
294 particular, *GATA4* exhibits high degree (*i.e.*, more targeted genes likely regulated
295 by GATA4) within the network, indicating important roles of GATA4 in TKI-
296 induced transcriptional dysregulation.

297 Mitochondrial biogenesis plays an important role during heart development,
298 and heart development is highly associated with mitochondrial dynamics,
299 biogenesis, and oxidative metabolism^{28,37-39}. A previous study showed that
300 genomic GATA4-occupied regions in mouse fetus (E11.5) are associated genes
301 related to “mitochondrial organization”, such as *Ppargc1a*⁴⁰, suggesting that
302 GATA4 may be one important regulatory factor for mitochondrial complex
303 assembly during prenatal development. In our study, the WGCNA analysis, the
304 motif enriched analysis, and the PECA analysis strongly suggest that *GATA4* is
305 likely to regulate genes involved mitochondrial complexes and biogenesis. Thus,
306 we hypothesize that TKIs induce mitochondrial dysfunction during CM
307 differentiation *via* inhibition of GATA4-mediated networks, ultimately leading to
308 dysfunctions in differentiated CMs. This led us to investigate the role of GATA4 in

309 CM differentiation upon exposure to TKI, and to examine whether CMs and their
310 mitochondrial function can be restored through overexpression of *GATA4*.

311

312 **Gain-of-function by GATA4 overexpression restores cardiomyocyte**
313 **functions in the presence of TKI exposure**

314

315 In order to better understand the role of GATA4 in TKIs induced cardiotoxicity
316 during differentiation, we created a lentiviral delivery-based dCas9/CRISPR-
317 activation (dCas9/CRISPRa) system based on previous studies^{41,42}, which
318 allows us to find out whether enhance of GATA4 expression will ameliorates TKI-
319 induced toxicity in differentiated CMs. The GATA4 overexpression *via* this
320 dCas9/CRISPRa system is doxycycline-inducible (Figure 4A and Supplemental
321 Figure 4A). Because our RNA-seq data showed that GATA4 expression was
322 down-regulated after day 6 upon exposure to TKI drugs (Supplemental Figure
323 4B), 2 µg/ml doxycycline was added daily to induce the expression GATA4 after
324 day 6 (Figure 4B-4D). In spontaneous calcium transient analysis, we found the
325 impaired Ca²⁺-handling signaling of TKI-treated CMs was restored by GATA4
326 induction, including increased beating rate and amplitude, faster calcium
327 handling as the time to peak, TD90, TD50, and decay tau were all decreased
328 (Figure 5A-5D). In addition, we also observed the disorganization of sarcomeres
329 in TKI treated CMs were reinstated by GATA4 expression (Figure 5E). These
330 results suggest that TKI-induced toxicity in CMs can be reversibly recovered by
331 enhancing GATA4 expression.

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333

334 **Overexpression of GATA4 restored mitochondrial function in differentiated**
335 **cardiomyocytes upon TKI exposure**

336

337 Integrative network analysis showed that GATA4 is involved in the regulatory
338 networks related to cardiac functions, metabolic process, and mitochondrial
339 function, and TKI-induced impairment of mitochondrial respiration. We therefore
340 investigated whether TKI-impaired mitochondrial respiration of CMs can also be
341 restored by enhancing GATA4 expression.

342 As expected, we observed overexpression of GATA4 during CM
343 differentiation improved basal and maximal respiration in differentiated CMs in
344 the presence of chronic exposure to the TKIs (Figure 6A-6C). This enhancement
345 of mitochondrial respiration by overexpression GATA4 was also observed in the
346 control group in the absence of drug exposure (Figure 6D). We also found that
347 overexpression of GATA4 promoted ATP production (Figure 6E) and
348 mitochondrial membrane potential (Figure 6F) in the CMs upon developmental
349 exposure to TKIs, and the most significant improvement was observed in
350 sunitinib-treated group. Moreover, chronic TKI exposure decreased branch size
351 of the mitochondria in differentiated CMs compared to control, which was
352 reversed by induction of GATA4 (Figure 6G-6O).

353 We next examined dynamics of mitochondrial DNA (mtDNA) levels during CM
354 differentiation, and found that mtDNA numbers increase from cardiac progenitor
355 stage (day 6) towards to the differentiating CM stages (Supplemental Figure 5A),
356 suggesting similarity in the increases of cardiac mitochondria during both *in-vivo*
357 and *in-vitro* cardiac differentiation^{38,43}. Chronic TKI exposure significantly
358 relatively lowered mtDNA copy numbers compared to control cells after day 6 of
359 differentiation compared to the control group (Supplemental Figure 5B-5C),
360 whereas overexpression of GATA4 increased the mtDNA copy numbers
361 (Supplemental Figure 5D), suggesting that overexpression of GATA4 promotes
362 mitochondrial biogenesis.

363 We explored the genome-wide GATA4-binding sites using ChIP-seq
364 experiments, and we found a global loss of GATA4-binding upon sunitinib
365 exposure compared to that in control group (Figure 7A). Genes regulated by
366 GATA4 are related to cardiac structure (“actin filament binding” and “PDZ domain
367 binding”), homeostasis of ion channels (“ion channel binding”), and signaling
368 pathways (“SMAD binding”). By contrast, sunitinib exposure attenuated the
369 enrichment of relevant GO terms in drug-treated CMs, consistent with loss of
370 GATA4-binding within these regions (Figure 7B), demonstrating sunitinib induced
371 adverse effects on cardiac dysfunction and sarcomere structure *via* inhibition of
372 transcriptional activity of GATA4.

373 Previous studies have showed that GATA4-binding DNA regions function as
374 enhancer regulatory elements for *Ppargc1a*, and overexpression of GATA4 can
375 increase *Ppargc1a* promoter activity^{40,44}. In the present study, specific analysis
376 of GATA4 occupancy near *PPARGC1A* identifies a GATA4 binding site
377 approximately 42-Kb upstream of *PPARGC1A* (Figure 7C and Supplemental
378 table 3). ChIP-seq experiments carried out in drug-treated CMs identifies a loss
379 of GATA4 occupancy at this putative *PPARGC1A* enhancer in sunitinib-treated
380 CMs compared with control (Figure 7C). Both mRNA and protein expression
381 levels of PGC-1 α were downregulated in differentiate CMs upon TKI exposure
382 and, conversely, upregulated by overexpression of GATA4 (Figure 7D and
383 Supplemental Figure 5E). Moreover, further integration of the current GATA4
384 ChIP-seq data with promoter-capture Hi-C data previously reported in analogous
385 day 20 CMs⁴⁵, we confirmed a direct interaction between the GATA4-binding site
386 with the *PPARGC1A* promoter anchor in CMs, consistent with long-range
387 enhancer-promoter regulation through DNA looping (Figure 7E). Given the
388 important roles of PGC-1 α in regulating mitochondrial biogenesis and functions,
389 altogether, this demonstrates that GATA4 binds to a *PPARGC1A*-enhancer and
390 directly influences the expression of PGC-1 α , thereby controlling mitochondrial
391 biogenesis and function at early stages.

392

393 **Maternal exposure to sublethal level of sunitinib induced developmental** 394 **defects in mouse fetus heart**

395

396 To evaluate the *in-vivo* toxicity of sunitinib during cardiac development, we
397 investigated the effects of maternal sunitinib exposure in mouse fetus.
398 Concentration of sunitinib used for daily intraperitoneal injection in female mouse
399 was 3mg/kg/day, which is proportional to the concentration used for human stem
400 cells in this study. Mice embryos were exposed to sunitinib from prenatal stages
401 (E0) towards neonatal stage (P0) (Supplemental Figure 6A). We observed that
402 maternal sunitinib exposure caused embryonic death (2 cases, Supplemental
403 Figure 6B) and moderate CHD-like histopathologic morphology (*i.e.*, thinner
404 myocardium and ventricular septal defect) in some of the surviving pups (around

405 10%) (Supplemental Figure 6C-6E). We also observed high level of
406 bioaccumulation of sunitinib in the blood of both female mice and newborns (P0),
407 and level of sunitinib in P0 newborns was 80% of that in female (Supplemental
408 Figure 6F). These results suggest that TKIs can accumulated in embryos by
409 maternal exposure and exert developmental toxicities during fetal development.

410

411

412 **DISCUSSION**

413

414 **Human stem cells as a cell-based platform to study developmental** 415 **cardiotoxicity**

416

417 The present study provides a new paradigm of using human stem cells to model
418 developmental exposure, so as to better understand the mechanisms underlying
419 drug-induced developmental cardiotoxicity. It has been reported that exposure to
420 various chemicals during pregnancy increases the risks for CHDs, and many of
421 these chemicals are able to cross the placental barrier ⁴⁶, such as heavy metals
422 (e.g., lead ⁴⁷ and cadmium ⁴⁸), pesticides ^{49,50}, arsenic ⁴⁸. In addition, a number of
423 medications are known to have high risks for congenital defects, such as
424 thalidomide ^{13,14} and retinoic acids ⁵¹⁻⁵³. Due to ethical restrictions curtailing the
425 use of human embryonic tissues in scientific researches, human stem cells have
426 provided us an alternative model to understand developmental processes in
427 humans ^{5,6}.

428

429 **TKIs and developmental cardiotoxicity**

430 TKI drugs have been used for cancer chemotherapy. However, they are known
431 to cause adverse effects in cardiovascular system in adults (*i.e.*, off-target
432 toxicity), including hypertension, left ventricular systolic dysfunction, QT
433 prolongation, and heart failure ⁵⁴⁻⁵⁷. As TKIs can cross the placenta to expose the
434 fetus during development ¹⁵, TKI-induced developmental cardiotoxicity has also
435 been reported in human ^{8-10,18} and animals ⁵⁸. On the other hand, mutation of
436 several targets for TKIs are associated with CHD, such as ABL Proto-Oncogene
437 1 (*ABL1*, targeted by imatinib) ⁵⁹, and platelet-derived growth factor receptor

438 alpha (*PDGFRA*, targeted by imatinib and sunitinib) ^{60,61}. All these reports
439 strongly suggested that maternal exposure of TKIs present high risks of
440 developmental cardiotoxicity and CHD.

441 The concentration of 250 nM used in this study was lower than plasma levels
442 of these TKI drugs achieved in clinical use ^{15,21-25}. Our results from modeling
443 stem cells provides direct evidences of cardiotoxicity derived from exposure at
444 this concentration, suggesting that sublethal level of TKIs (especially sunitinib)
445 still exert mild toxicity in developing heart and cause dysfunctions (*such as*
446 congenital arrhythmia), even though cardiac development still successfully
447 proceeds to four-chamber heart structures in the fetus. The bioaccumulation and
448 cardiotoxicity from maternal sunitinib exposure was evident in the *in-vivo* mouse
449 study, and the potential long-term adverse effects of sunitinib in offspring need to
450 be characterized in future studies.

451

452 **GATA4 as a key regulator in cardiotoxicity from exposure to anticancer** 453 **drugs**

454

455 Previous studies showed that overexpression of GATA4 in early developmental
456 stages promote CM differentiation ^{62,63}. Although overexpression of GATA4 in the
457 neonatal rat causes a mild form of cardiac hypertrophy at six months of age ⁶⁴,
458 GATA4 acts as important regulator for cardiac angiogenesis and myocardial
459 regeneration in neonatal mice ^{65,66}. These studies suggest the multiple roles of
460 GATA4 in both early and postnatal cardiac developmental stages. Moreover,
461 GATA4 has been shown to play protective roles in cardiotoxicity from exposure
462 to anthracycline anticancer agents (*e.g.*, doxorubicin), and overexpression of
463 GATA4 can reduce doxorubicin-induced apoptosis and increase the survival of
464 CMs ^{67,68}. Regarding effects of TKIs on GATA4, a single study by Maharsy *et al.*
465 ⁶⁹ showed that down-regulation of GATA4 in heart is associated with dietary
466 imatinib exposure in mice. Whether GATA4 could exert protective roles in TKI-
467 treated CMs was unknown.

468 In our study, both gene and protein expression, as well as DNA-binding of
469 GATA4, were attenuated upon exposure to TKIs, demonstrating that GATA4 was

470 targeted by TKIs, similar to doxorubicin, consequently leading to dysregulation of
471 GATA4-mediated transcription networks and cardiac dysfunction in differentiated
472 CMs. In addition, we also observed variation in inhibition of TFs among different
473 TKI drugs. For instance, sunitinib exerted the most significant adverse effects on
474 genomic binding of GATA4, compared to that of imatinib and vandetanib; while
475 vandetanib exerted more significant effects on MEF2s (Supplemental Figure 3A).
476 Giving the obvious toxic effects (e.g., sarcomeric disarrangement, mitochondrial
477 size, and ATP production) on sunitinib-treated group, this demonstrates that
478 GATA4 acts as important role in protection of sunitinib-induced developmental
479 toxicity.

480

481 **Mitochondria, GATA4 and cardiac development**

482 It is known that heart development undergoes metabolic switch from glycolysis to
483 OXPHOS²⁶. Mitochondrial biogenesis and maturation occur during prenatal
484 stages of mice when glycolysis is dominant. During cardiac development,
485 increases in both cardiac mitochondrial number and inner folding membranes
486 (*i.e.*, cristae) are shown from E8.5 in mice, and mitochondrial complexes started
487 to assemble at E11.5; from E13.5 to P0 in mice, mitochondria are functionally
488 matured, thus the newborns can generate ATP relied on OXPHOS at postnatal
489 stages^{38,39,43,70}.

490 TKI drugs haven been shown to induced mitochondrial dysfunction, such as
491 respiration or membrane potential, in variable cell types⁷¹⁻⁷³, suggesting that
492 disruption of mitochondrial biogenesis and functions upon exposure to TKI can
493 cause developmental problems in heart development. A previous study by
494 Kasahara *et al.*⁷⁴ showed that depletion of mitochondrial fusion proteins
495 Mitofusin 1 and 2 (MFN1 and MFN2) arrested heart development in mouse
496 embryos and CM differentiation from embryonic stem cells, and this event was
497 accompanied with the decreased GATA4 levels, suggesting a potential
498 relationship between mitochondrial biogenesis and GATA4 during heart
499 development. However, given both GATA4 and mitochondrial biogenesis are
500 well-known to control heart development, the relationship between GATA4 and

501 mitochondria has not been well-documented. Our study showed that GATA4
502 occupies enhancer regions for *PPARGC1A* in differentiating CMs, corroborating
503 a previous study using the mouse fetus⁴⁰. Induction of *PPARGC1A* expression
504 *via* GATA4 overexpression can be considered as a potential approach, which is
505 capable of restoring the mitochondrial respiration and morphology from TKI
506 exposure.

507 Besides GATA4, the TEADs and MEF2s families are also known to regulated
508 cardiac development⁷⁵⁻⁷⁷; and these TFs are reported to be involved in
509 regulating mitochondrial biogenesis *via* activation of PGC1 α expression⁷⁸⁻⁸⁰.
510 Previous studies showed that DNA-binding of TEADs and MEF2s co-occupies
511 with that of GATA4 in mouse fetus, indicating interactions among these TFs in
512 the regulation of genes controlling heart development and mitochondrial
513 biogenesis^{40,81,82}. In the present study, the TKI-treated groups exhibited
514 decreased DNA-binding signals of GATA4, TEADs and MEF2s than that in
515 control group, suggesting that TKIs exposure also targeted TEADs or MEF2s in
516 differentiating CMs. Given the importance of these TFs in cardiac differentiation
517 and mitochondria, combinatorial overexpression of these TFs could be
518 considered as a more powerful approach to rescue CMs and mitochondrial
519 function from developmental TKI-exposure.

520 In sum, we demonstrated cardiotoxicity and mitochondrial dysfunction from
521 developmental exposure to TKI exposure during CM differentiation derived from
522 human stem cells. We demonstrate that TKI-induced toxicity was also observed
523 in mice study. Induction of GATA4 with CRISPRa has restored functions and
524 morphology of differentiated CMs and mitochondria. The present study
525 established an integrated stem cell-omics platform to investigate important
526 mechanisms underlying developmental heart defects from chemical exposure.
527 The novel crosstalk mechanism between GATA4 and mitochondria identified
528 from this study will help us to develop therapeutic solutions to minimize toxicity
529 from maternal drug exposure during early cardiac development.

530

531

532 **EXPERIMENTAL PROCEDURES**

533

534 **Chemicals**

535 Imatinib Mesylate (S1026, Selleck Chemicals, TX), Sunitinib (RS046, BIOTANG
536 Inc, MA), vandetanib (RS051, BIOTANG Inc), tacrolimus (RS047, BIOTANG Inc),
537 Mycophenolate Mofetil (S1501, Selleck Chemicals), and thalidomide
538 (ICN15875380, Fisher scientific) were ordered and dissolved in DMSO as stocks.

539

540 **Cell culture, cardiomyocyte differentiation and chemical exposure**

541 The H1-hESCs (RRID: CVCL_9771) were obtained from the Stem Cell Core
542 Facility of Genetics, Stanford University. The pluripotent cells were grown in
543 Matrigel (Corning)-coated 12-well plates in Essential 8 Medium (Thermo Fisher
544 Scientific) at 37°C incubators (5% CO₂). Cardiomyocyte differentiation was
545 initiated using a monolayer differentiation chemically defined method²⁰. Drug
546 exposures were conducted using two experimental designs, including: Exposure
547 I, a brief early exposure design, in which the TKI drugs were added from day 0 to
548 day 6 (cardiac progenitor); Exposure II, in which cells were exposed to TKI drugs
549 throughout the entire differentiation process until CMs were collected for
550 analyses (Figure1A). To further increase cardiomyocyte purity, the differentiated
551 cells were subjected to subsequent glucose starvation using non-glucose-
552 supplemented RPMI/B27 medium twice (two days per time) to decrease non-
553 cardiomyocyte cells, since cardiomyocytes are more tolerant to glucose
554 starvation⁸³. Differentiated beating cardiomyocytes were harvested by TrypLE
555 Select 10X (Thermo Fisher Scientific). Regarding imaging and functional
556 analyses, cells were re-plated with RPMI/B27 supplemented with 10% FBS and
557 10 μM ROCK inhibitor.

558

559 **Immunostaining of sarcomere of differentiated cardiomyocytes**

560 For immunostaining imaging of sarcomere structure, the differentiated
561 cardiomyocytes were re-plated in Nunc™Lab-Tek™II glass-bottomed 8-chamber
562 glass slides (Thermo Fisher), and then cells were fixed and permeated in the
563 plate using a Human Cardiomyocyte Immunocytochemistry Kit (Thermo Fisher

564 Scientific). The primary antibodies included: rabbit anti-cardiac troponin T
565 (Abcam, ab45932, RRID: AB_956386) and mouse anti- α -actinin (sarcomeric)
566 (Sigma-Aldrich, A7811, RRID: AB_476766). The secondary antibodies included:
567 goat anti-rabbit IgG, Alexa Fluro 594 (Thermo Fisher Scientific, R37117, RRID:
568 AB_2556545) and goat anti-mouse IgG, Alexa Fluro 488 (Thermo Fisher
569 Scientific, A-11001, RRID: AB_2534069). The images were taken using Leica
570 DMi8 Microsystems and Zeiss LSM710 inverted confocal microscope, and then
571 the images were processed using the Fiji software (RRID: SCR_002285).

572

573 **Spontaneous Ca²⁺ transient imaging and measurement in cardiomyocytes.**

574 Differentiated beating cardiomyocytes were dissociated by TrypLE Select 10X
575 (Thermo Fisher Scientific) and 50,000 of cells were re-plated in Matrigel (BD
576 Bioscience) pre-coated 8-well LAB-TEK® II cover glass imaging chambers
577 (Thermo Fisher Scientific). Cells were recovered for 3-4 days after seeding until
578 beating normally. Calcium imaging was performed as previously described⁸⁴.
579 Briefly, cells were loaded with 5 μ M Fluo-4 AM in Tyrode's solution (140 mM
580 NaCl, 1 mM MgCl₂, 5.4 mM KCl, 1.8 mM CaCl₂, 10 mM glucose, and 10 mM
581 HEPES pH = 7.4 with NaOH at RT) for 5-10 min at 37°C. After washing with pre-
582 warmed Tyrode's solution 3 times, cells were immersed in Tyrode's solution for 5
583 min prior to imaging. Spontaneous calcium signaling in cardiomyocytes was
584 sampled by confocal microscopy (Carl Zeiss, LSM 510 Meta, Göttingen,
585 Germany) with a 63X oil immersed objective (Plan-Apochromat 63x/1.40 Oil DIC
586 M27). Signaling was captured in line-scanning mode (512 pixels X 1920 lines) at
587 a speed of 3.2 μ s/pixel. For analysis of the data, a custom-made script based on
588 IDL (Interactive digital language) was used. Extracellular background signal was
589 subtracted from calcium signals, and the calcium signal was normalized to the
590 intracellular basal line (F₀). Transient amplitude was expressed as $\Delta F/F_0$. Decay
591 Tau (mS) was calculated by mono exponential curve fitting.

592

593 **Flow cytometry and analysis**

594 Differentiated cardiomyocytes (day20, around 50,000 cells) were collected for
595 flow cytometry. The cells were washed with DPBS buffer (Thermo Fisher
596 Scientific), and then were fixed and permeabilized using Cytotfix/Cytoperm (BD
597 Biosciences). Afterwards, the cells were labeled with rabbit anti-cardiac Troponin
598 T (*i.e.* cardiac TNNT2) antibody (Abcam, ab45932, RRID: AB_956386) (1:100
599 dilution in Perm/Wash buffer, BD Biosciences), and then labeled with goat anti-
600 rabbit IgG (Alexa Fluor 488 conjugated, Thermo Fisher Scientific, A11034, RRID:
601 AB_2576217) secondary antibody (1:200 dilution). Ice-cold DPBS (with 1% FBS)
602 was used as the flow cytometry buffer for re-suspending cells. Flow cytometry
603 was performed using a FACSAria II cytometer (BD Biosciences). The data were
604 analyzed using FlowJo software (version 10.1). The events were first gated to
605 filter dead cells and debris. Troponin T-positive cells were defined as cells having
606 a fluorescence density greater than the isotype control.

607

608 **RNA-seq and data analysis**

609 Cells at Day 0, day 6, and day 20 were collected for RNA-seq. For each
610 treatment and each time-point, cells from two independent differentiation wells
611 were used as two biological replicates. Total RNA was extracted from the same
612 number of cells among each group using QIAzol lysis reagent (Qiagen), and
613 RNA was then subjected to DNase I digestion and purified using a miRNeasy
614 Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was
615 checked with a NanoDrop, and only samples with a ratio of 260/280 between 2.0
616 - 2.1 were subsequently used for ribosome depletion. Purified RNA (2.5 µg) was
617 used for ribosome depletion using a Ribo-Zero™ Gold Kit (Human/Mouse/Rat)
618 (Epicentre Biotechnologies) according to the manufacturer's instructions. The
619 integrity of ribosome-depleted RNA was assessed using an Agilent RNA 6000
620 Pico Assay kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA-seq
621 libraries were constructed using ScriptSeq™ v2 RNA-Seq Library Preparation
622 kits (Epicentre Biotechnologies) according to the manufacturer's instructions. The
623 concentration of the library was measured with a Qubit Fluorometer (Thermo
624 Fisher Scientific) and the size was determined using an Agilent High Sensitivity

625 DNA kit on an Agilent 2100 Bioanalyzer. All RNA-seq libraries were sequenced
626 by HiSeq4000 sequencers (Illumina) with 2 x 101 cycles.

627 The raw RNA-seq raw data were trimmed to remove the adapter sequences
628 (including GATCGGAAGAGCACACGTCTGA and
629 AGATCGGAAGAGCGTCGTGTAG) with command-line tool cutadapt (1.8.1).
630 Then the trimmed files were aligned with Tophat (version 2.0.9) to GRCh37/hg19
631 *Homo sapiens* reference genome. The human gene symbols and their raw
632 counts were calculated using the HTSeq⁸⁵ (version 0.6.1p1) package in Python
633 with the hg19 *Homo sapiens* gtf file. Differential gene-expression analysis was
634 performed using the edgeR package in R, and the normalization was performed
635 using a trimmed mean of M-values (TMM) method across all samples⁸⁶. The
636 Gene Ontology (GO) enrichment analysis was performed using on-line tools
637 DAVID (version 6.8) (<https://david.ncifcrf.gov/summary.jsp>) and the Gene
638 Ontology Resource (<http://geneontology.org>). The Gene Ontology (GO)
639 enrichment analysis of differentially expressed genes was performed using
640 DAVID (<https://david.ncifcrf.gov>).

641

642 **ATAC-seq and data analysis**

643 The ATAC-seq protocol developed was used for the chromatin accessibility
644 profiling³⁰. Cells at day 6 and day 20 were collected for ATAC-seq. For each
645 treatment and each time-point, cells from two independent differentiation wells
646 were used as two biological replicates. For each sample, 50,000 cells were
647 collected and pelleted by centrifugation for 15 min at 500 g and 4°C. Cell pellets
648 were washed once with ice-cold 1x PBS and then pelleted again by
649 centrifugation at the previous settings. Cell pellets were re-suspended in 25 µl of
650 ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1%
651 Igepal CA-630), and nuclei were pelleted by centrifugation for 30 min at 500g,
652 4°C. Supernatants were discarded and nuclei were re-suspended in 50 µl
653 reaction buffer (2.5 µl of Tn5 transposase and 25 µl of TD buffer from a Nextera
654 DNA Library Prep Kit from Illumina, and 22.5 µl nuclease-free H₂O). The reaction
655 was incubated at 37°C for 30 min, and subsequently the reaction mixture was

656 purified using MinElute PCR Purification Kit (Qiagen). The purified transposed
657 DNA was amplified with NEBNext High-Fidelity 2 X PCR Master Mix (New
658 England Biolabs) and custom-designed primers with barcodes.³⁰ Gel
659 electrophoresis was used to remove primer dimers from the PCR products with
660 2% E-Gel EX Agarose Gels (Thermo Fisher Scientific), and then the PCR
661 products were purified using QIAquick PCR Purification Kit (Qiagen). DNA
662 concentration was measured with a Qubit Fluorometer (Thermo Fisher Scientific)
663 and library sizes were determined using Agilent High Sensitivity DNA kit on
664 Agilent 2100 Bioanalyzer. The ATAC-seq libraries were sequenced with a HiSeq
665 4000 sequencer (Illumina) with 2 X101 cycles, and the sequencing quality control
666 was performed by Stanford Center for Genomics and Personalized Medicine.

667 The raw data were trimmed to remove the adapter sequences
668 (CTGTCTCTTATACACATCT) with command-line tool cutadapt (1.8.1), and then
669 the trimmed files were mapped to the human genome (hg19) using Bowtie2
670 (2.1.0) with default parameters. Read pairs, which were aligned concordantly to
671 the genome and had a mapping quality greater than 10, were kept for following
672 analysis. Read pairs mapped to mitochondria DNA were discarded. Redundancy
673 read pairs from PCR amplification were also removed afterward using Picard
674 tools (version 1.79). The finally filtered bam files were converted into normalized
675 TDF files using igvtools (2.26.0) for visualization of the peaks in the IGV
676 software. Open accessible-regions for each library were defined by the peaks
677 called by MACS2 (2.1.0) with the parameters “-g hs --nomodel --shift 0 -q 0.01”.
678 Peaks located at blacklist genomics regions were removed using bedtools
679 (2.25.0). These tracks shows artifact regions that tend to show artificially high
680 signal and were identified by the ENCODE and modENCODE consortia⁸⁷. The
681 filtered bam files and filtered bed files were used to generate differential peaks
682 (FDR<0.05) using the DiffBind package in R^{88,89}. The annotations of the peaks
683 were achieved using ChIPpeakAnno, org.Hs.eg.db, and EnsDb.Hsapiens.v75
684 packages in R.

685

686 **Motif analysis**

687 For transcription factor motif analysis, the sequences of differential peaks (+/-
688 100 bp) were used for the motif analysis. MEME-chip⁹⁰, as part of MEME Suite of
689 motif-based sequence analysis tools (<http://meme.nbcr.net>) was used for the
690 comprehensive motif analysis. The enriched (E value < 0.05) *de novo* DNA-
691 binding motifs were identified by MEME-ChIP Discriminative DNA motif discovery
692 (DREME), which uses Fisher's Exact Test to test the significance. Transcription
693 factors for each enriched motif were determined using Tomtom against the
694 known motif databases (e.g. JASPAR). The set of sequences for individual
695 matched motif were determined by Find Individual Motif Occurrences (FIMO)
696 within the MEME suite⁹¹. Homer (version 4.10) was used for motif density
697 analysis for TF of interests⁹². Plotting of normalized tag around motif was
698 performed using ngsplot⁹³.

699

700 **ChIP-seq and analysis**

701 Antibody of GATA4 (Abcam, ab124265, RRID: AB_11000793) was used for
702 ChIP-seq in this study. For each group, chromatin immunoprecipitation was
703 performed using approximately 1×10^7 cells. Cells were first cross-linked with 37
704 % formaldehyde for 10 min at room temperature, and formaldehyde was
705 quenched for 5 min by glycine with a final concentration of 0.125 M. Chromatin
706 was broken into small pieces with an average size of 0.5-2 kb using the Bioruptor
707 (Diagenode). The sonicated chromatin was then incubated with 5 μ g of primary
708 antibodies overnight at 4°C. A small portion (10%) of chromatin without antibody
709 incubation was kept as input DNA for each ChIP reaction. Subsequently, 6 μ g of
710 GATA4 antibody with 75 μ l of Dynabeads Protein A/G were added and incubated
711 overnight at 4°C with overhead shaking. Magnetic beads were then washed
712 away and chromatin was eluted. Crosslink was reversed and precipitated DNA
713 was purified and resuspended in nuclease-free water. Sequencing libraries of
714 immunoprecipitated DNA and input DNA were constructed according to an
715 Illumina DNA library preparation protocol. Subsequently, ChIP-seq libraries were
716 loaded to an Illumina HiSeq 4000 platform for deep sequencing.

717 The raw data were trimmed to remove the adapter sequences (including
718 AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC and
719 AGATCGGAAGAGCG-
720 TCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT) with
721 command-line tool cutadapt (1.8.1). The rest steps are the same to the ATAC-
722 seq data analysis. Open accessible-regions for each sample were defined by
723 subtracting input background by MACS2 (2.1.0) with the parameters “-t sample -c
724 input”. Differential analysis was performed by DiffBind package in R ^{88,89}. The
725 annotations of the peaks were achieved using annotatePeaks.pl of the Homer
726 software (version 4.10) ⁹². PPARGC1A-centric promoter loops were selected
727 from promoter-capture Hi-C loops called in iPSC-derived cardiomyocytes ⁴⁵.
728 Processed loop calls were downloaded from ArrayExpress accession number E-
729 MTAB-6014, and three replicate cardiomyocyte loop calls (capt-
730 CHiCAGO_interactions-CM) were visualized using the Sushi R/Bioconductor
731 package for genomic data visualization ⁹⁴. Loops intersecting the GATA4 peak
732 coordinates (chr4:23374967-23375240) are colored red.

733

734 **Network analyses**

735 Weighted correlation network analysis (WGCNA) was used to identified gene co-
736 expression networks ⁹⁵. The log-transformed values of the normalized counts of
737 all transcripts were used to perform weighted gene co-expression network
738 analysis using WGCNA package in R. Only transcripts with a sum of values
739 across all samples that were larger than 10 and a variance larger than 0 were
740 used for the network analysis. For integrative network analysis, a statistical
741 approach based on the paired expression and chromatin accessibility (PECA)
742 was used for modeling gene regulation ^{31,32}.

743

744 **Mitochondrial function and morphology analyses**

745 Several dyes were used for investigation of mitochondrial function. For
746 mitochondrial morphology analysis, 200 nM of MitoTracker® Green FM dye
747 (Thermo Fisher Scientific, M7514) was used for staining live mitochondria. The

748 images were taken using Zeiss LSM710 inverted confocal microscope, and then
749 mitochondrial morphology was analyzed using the Fiji software⁹⁶. 100 nM of
750 tetramethylrhodamine (TMRM) (Thermo Fisher Scientific, I34361) was used for
751 measuring mitochondrial membrane potential. Fluorescence of these dyes was
752 measured using a Tecan M1000 multimode plate reader (Tecan Systems, Inc.,
753 CA).

754

755 **Mitochondrial DNA dynamics analysis**

756 Human mitochondrial to nuclear DNA ratio kit (Takara) was used to assess
757 mitochondrial DNA content. Two separate primer pairs were used to generate
758 nuclear-mitochondrial DNA content ratios. SLCO2B1 and SERPINA1 were used
759 as nuclear genes, while ND1 and ND5 were used as mitochondrial genes. Two
760 genes for both nuclear and mitochondrial DNA were used as an average to
761 prevent outliers. A mathematical ratio was generated to determine the
762 mitochondrial DNA content of each sample.

763

764 **Mitochondrial respiratory activity assay**

765 The mitochondrial respiratory activity in cardiomyocytes was analyzed by
766 mitochondrial stress test using a Seahorse XFp Extracellular Flux Analyzer
767 (Agilent, CA). 45,000 of cells were plated into an XFp culture plate (Agilent) with
768 RPMI/B27 supplemented with 10% FBS and 10 μ M ROCK inhibitor. After 48h of
769 recovering, mitochondrial stress test was performed using a Seahorse XF Cell
770 Mito Stress Test kit (Agilent) according to the manufacture manual. Briefly, one
771 day prior to the experiment, the XFp sensor cartridge was hydrated in XF
772 calibrator solution and incubated overnight at 37 °C in a non-CO₂ incubator. One
773 hour prior to the experiment, the cells were incubated at 37 °C (non-CO₂) in 200
774 μ l of Seahorse assay medium, containing XF base medium supplemented 1 mM
775 pyruvate, 2 mM glutamine, and 10 mM glucose (pH 7.4). OCR was measured
776 with sequential injections of 2 μ M oligomycin, 2 μ M FCCP and each 0.5 μ M of
777 rotenone/antimycin A. Data were normalized to cell numbers for each wells,

778 which were re-calculated using a TC20 Automated cell counter (Bio-Rad, CA)
779 with trypan blue solution (4%)

780

781 **ATP production measurement**

782 ATP production in differentiated cardiomyocytes were evaluated using eATP
783 Colorimetric/Fluorometric Assay Kits (BioVision Inc, CA). Briefly, 1×10^6 cells
784 were homogenized in 110 μ l ATP Assay buffer, and then proteins were removed
785 using ice-cold 20 μ l perchloric acid from a Deproteinizing Sample Preparation Kit
786 (BioVision Inc, CA). After incubation on ice for 5 min, the sample were spun
787 down at 13000 g for 2 min. Supernatant (about 120 μ l) was collected and
788 combined with 20 μ l of ice-cold neutralization solution. The final solution was
789 measured in a 96-well plate according to the protocol, and the fluorometric assay
790 was conducted in a Tecan M1000 multimode plate reader (Excitation/Emission =
791 535/587 nm).

792

793 **Plasmids for of CRISPR-mediated gene activation**

794 Two-vector system was used to establish doxycycline-inducible dCas9-VP64-
795 mediated gene activation cells, *i.e.*, lentiGuide-Puro plasmid (a gift from Feng
796 Zhang, Addgene #52963, RRID: Addgene_137729⁴²) expressed sgRNA and
797 pHAGE TRE-dCas9-VP64 plasmid (a gift from Rene Maehr & Scot Wolfe,
798 Addgene plasmid #50916, RRID: Addgene_50916⁴¹) expressed inactive version
799 of Cas9 (dCas9) fused to a VP16 tetramer activation domain (VP64). For the
800 sgRNA constructs, the 20-bp oligo of sgRNA was designed based on upstream
801 of TSS region of GATA4, and then were cloned into the *BsmBI* site of the
802 lentiGuide-Puro plasmids. The sgRNAs were designed using the on-line tool
803 (<http://crispr-era.stanford.edu>) and their expression are driven by U6 promoter.
804 The sgRNA sequences of all plasmids were confirmed by Sanger sequencing:
805 sg_GATA4_1: GAACCCAATCGACCTCCGGC (-258bp to TSS of GATA4);
806 sg_GATA4_2: GGTGATTCCCCGCTCCCTGG (-229bp to TSS of GATA4). Both
807 sgRNAs were confirmed to induce GATA4 expression during early

808 cardiomyocyte differentiation (Supplemental Figure 4A). The sg_GATA4_1 was
809 selected in the study to investigate outcomes from overexpression of GATA4.

810

811 **Lentivirus preparation and infection in stem cells**

812 HEK293T (ATTC, Cat# CRL-3216, RRID: CVCL_0063) cells were maintained in
813 6-well plates with Dulbecco's Modified Eagle Medium (Gibco) supplemented with
814 10% fetal bovine serum and Penicillin Streptomycin. Packaging plasmids (pVSVg
815 and psPAX2), lentiGuide-Puro plasmids (with sgRNAs) or TRE-dCas9-VP64
816 plasmids, Opti-MEM (Thermo Fisher Scientific), and X-tremeGENE 9 DNA
817 transfection reagent (Sigma-Aldrich) were used to transfect HEK293T cells
818 according to the manufacturer's instructions. Media supernatant containing virus
819 particles were filtered with 0.45 μ M filter and further concentrated using Lenti-X
820 according to the manufacturers' protocol. The stem cells were firstly infected with
821 the pHAGE TRE-dCas9-VP64 plasmids with 300 μ g/ml of G418 for selection;
822 and then they were secondly infected with the lentiGuide-Puro plasmids with 10
823 μ g/ml of puromycin for selection. Regarding lentiviral infection, 2 μ g/ml of
824 polybrene was used.

825

826 **Real-time QPCR**

827 cDNA was synthesized from 1 μ g of total RNA using a SuperScript VILO cDNA
828 Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's protocol.
829 PCR amplification was performed with a QuantStudio™ 6 Flex Real-Time PCR
830 System (Thermo Fisher Scientific) in 20- μ l reactions using 1 μ l of cDNA (10 ng of
831 total input RNA), 200 nM of each forward and reverse primer and 1X *Power*
832 SYBR Green PCR Master Mix (Applied Biosystems). The real-time PCR program
833 consisted of 1 cycle of 95°C for 5 min; and 40 cycles of 95°C for 15 s, 60°C 30 s
834 and 72°C for 30 s. The β -actin was used as a normalizing gene, and relative
835 gene expression data were calculated using the $\Delta\Delta$ Ct method⁹⁷. Primers used
836 were ordered from Qiagen, including GATA4 (QT00031997), PPARGC1A
837 (QT00095578), and β -actin (QT00095431).

838

839 **Western blot**

840 The cells were harvested in RIPA lysis buffer (EMD Millipore, CA) contain one
841 tablet of Pierce™ protease and phosphatase inhibitor (Thermo Fisher Scientific),
842 and the proteins were purified using a Branson Digital Sonifier homogenizer
843 (Branson Ultrasonics, CT). 20 µg of protein from each sample was separated on
844 NuPAGE 4-12% Bis-Tris protein gels (Thermo Fisher Scientific) and transferred
845 to nitrocellulose membranes (Thermo Fisher Scientific). The protein-bound
846 membranes were blocked with 5% of blotting-grade blocker (Bio-Rad) in PBST
847 for one hour at room temperature and incubated with a primary antibody in 5% of
848 blotting-grade blocker in PBST overnight at 4°C. After washing with PBST buffer,
849 the membranes were incubated with horseradish peroxidase (HRP)-conjugated-
850 secondary antibody for 1h at room temperature. The membranes were
851 developed with SuperSignal West Femto Maximum Sensitivity Substrate
852 (Thermo Fisher Scientific) and exposed on a ChemiDoc Touch imaging system
853 (Bio-Rad) for imaging. The primary antibody used in this study included mouse
854 anti-GATA4 (R&D systems, MAB2606, RRID: AB_2108599), rabbit anti-PGC1
855 alpha antibody (Abcam, ab54481, RRID: AB_881987), rabbit anti-HA-tag (Cell
856 Signaling Technology, #5017, RRID: AB_10693385), and mouse anti-beta-actin
857 (Thermo Fisher Scientific, MA5-15739, RRID: AB_10979409). The secondary
858 antibodies include HRP-conjugated-horse anti-mouse IgG (Cell Signaling
859 Technology, 7076S, RRID: AB_330924) and HRP-conjugated-goat anti-rabbit
860 IgG (Thermo Fisher Scientific, 32460, RRID: AB_1185567).

861

862 **Maternal TKI exposure in mice model**

863 The Stanford Institute of Medicine Animal Care and Use Committee approved all
864 protocols. 6-week-old female C57BL/6 mice purchased from Jackson Laboratory
865 (Bar Harbor, ME) were used. Female and male were housed as a 1:1 ratio in a
866 single cage in the afternoon (~4 pm). In the following morning (designated as
867 E0.5), females with vaginal plugs were injected with sunitinib and saline as
868 control. TKIs or saline were injected intraperitoneally every other day towards
869 E18.5. The doses for each drug per female mouse (~20 g) per injection (in 200

870 μ L saline) was as follows: sunitinib and vandetanib, 100 μ g; Imatinib, 1.5 mg. In
871 the morning of E19.5, the plasma from newborns (P0) and their mom were
872 collected by the following protocol. Blood samples were collected directly into
873 EDTA-treated tubes. The tubes were shaken gently, but thoroughly afterward.
874 Samples were then centrifuged at 20-24 °C at 4500 g for 10 min. Then the
875 plasma was transferred and aliquoted into pre-cooled Eppendorf tubes without
876 aspirating blood cells. Plasma samples were frozen immediately on dry ice and
877 stored at -80 °C.

878

879 **Detection of Drug Exposure by LC-MS**

880 The detailed methods used for metabolomic sample preparation, data
881 acquisition, and analysis have been described previously⁹⁸. Briefly, plasma
882 metabolites were extracted with organic solvent for LC-MS analysis. Samples
883 were separated using RPLC (Zorbax-SB-aq column 2.1 x 50mm, 1.7mm, 100Å;
884 Agilent) and collected in positive ion mode on a Thermo Q Exactive HF mass
885 spectrometer (Thermo Fischer Scientific). Raw data was processed with
886 Progenesis QI 2.3 software (Water, Milford, MA, USA) to align and quantify
887 chromatographic peaks. Imatinib (m/z: 494.2663) and sunitinib (m/z: 399.2191)
888 were identified by accurate mass, retention time, MS/MS fragmentation, and
889 quantified relative to standard curves.

890

891 **Statistical analysis**

892 Statistical analysis was performed using GraphPad Prism 8.4 (GraphPad
893 Software, Inc., San Diego, CA, RRID: SCR_002798). Nonparametric T-test was
894 used to compare data between two groups. Data are reported as means \pm
895 standard error of the mean (SEM).

896

897 **Data Availability**

898 The RNA-seq, ATAC-seq, and ChIP-seq data generated for this work have been
899 deposited in NCBI Gene Expression Omnibus, and they are accessible numbers
900 are GSE149586 for RNA-seq, GSE149589 for ATAC-seq, and GSE149591 for
901 ChIP-seq.

902 **FIGURE LEGENDS**

903

904 **Figure 1. Developmental exposure to sublethal level of TKIs induced**
905 **disarrangement of sarcomere and alteration in Ca²⁺-handling of**
906 **cardiomyocytes. (A)** Experimental design of drug exposure strategies. H1-
907 ESCs were differentiated to cardiomyocytes upon chronic exposure to sublethal
908 of drugs following two exposure designs: Exposure I, the TKI drugs were added
909 from day 0 to day 6 (cardiac progenitor); Exposure II, the cells were exposure to
910 TKI drugs till the differentiated CMs were collected for analyses. **(B)** Analysis of
911 the efficiency of CM differentiation with flow-cytometry of TNNT2-positive cell
912 populations, no significant difference was observed between control (0.1%
913 DMSO) and imatinib groups. More results of other groups are shown in
914 Supplemental Figure 1B. **(C)** Immunostaining of differentiated cardiomyocytes
915 with α -actinin (green), TNNT2 (red), and DAPI (blue). Developmental exposure to
916 TKIs caused disorganization of sarcomere structures of cardiomyocytes from
917 Exposure II. **(D)** Representative Ca²⁺-handling recording from control and
918 imatinib-treated cardiomyocytes. **(E-G)** Bar charts represent developmental
919 exposure to TKIs caused significant alternations of Ca²⁺-handling properties,
920 including decreases in beating rate and amplitude, and increased in time to peak,
921 TD90/50, and decay tau. The *p* values were calculated by nonparametric T-test
922 between control and TKI-treated groups. I, imatinib; V, vandetanib; S, sunitinib; +,
923 Exposure I; ++, Exposure II.

924

925 **Figure 2. Transcriptomic analysis revealed correlation between**
926 **transcriptional regulatory network and mitochondrial function. (A)** Weighted
927 gene co-expression network analysis (WGCNA) revealed modules based on
928 transcriptomic profiles from differentiated CMs (day20) in control and drug-
929 treated conditions. **(B)** The heatmap represents the gene expression pattern of
930 the module 1, using the normalized counts by trimmed mean of M-values (TMM)
931 normalization method. I, imatinib; V, vandetanib; S, sunitinib; Th, thalidomide; Ta,
932 tacrolimus; and MF, mycophenolate. Clustering analysis of the transcriptomic
933 profile revealed that TKI drug-treated cardiomyocytes exhibited high
934 concordance between Exposure I (+) and Exposure II (++) (except Exposure I for
935 imatinib) **(C)** Statistically enriched (FDR<0.05, red line indicated) gene ontology
936 (GO) terms of genes in the module 1 (FDR<0.05, red line indicated). **(D)**
937 Representative nodes from module 1, including transcription factor controlling
938 heart development, cardiac genes, and metabolic and mitochondrial related
939 genes. **(E)** Bar chart shows the regression coefficients of different drugs when
940 regressing the mean gene expression of module 1 on the drug usage. The data
941 are normalized to DMSO. **(F)** Evaluation of mitochondrial oxygen consumption
942 rate (OCR) in cardiomyocyte upon exposure to TKIs during differentiation. The
943 data are normalized by cell numbers. **(G)** ATP production was significantly
944 inhibited in differentiated cardiomyocytes by developmental exposure to TKIs.
945 Data are reported as means \pm standard error of the mean (SEM). The
946 corresponding *p* values were calculated by nonparametric T-test between control
947 and TKI-treated groups.

948

949 **Figure 3. Integrative network analysis revealed strong interactions between**
950 **transcription factors and mitochondrial genes. (A)** Genome-wide landscape
951 of chromatin accessibility in differentiated cardiomyocytes between DMSO (0%
952 and 0.1%), and TKIs. The red box indicates the down-regulated chromatin
953 accessibility in the TKI-treated cardiomyocytes. High correlation between 0%
954 DMSO and 1% DMSO indicated that DMSO did not cause aberrations in
955 chromatin accessibility. **(B)** Enriched *de novo* motifs (E value <0.001) were
956 discovered using MEME-chip based on down-regulated chromatin accessibility in
957 TKI-treated cells. **(C)** Motif density plots of representative transcription factors.
958 **(D-F)** ATAC-seq peak signals (+/- 1000 bps) arounds submits of the motifs for
959 GATA4 in TKI-treated cells compared with DMSO-treated cells. Binding
960 intensities are shown as sequencing depth-normalized tag count. These figures
961 are partial data of Supplemental Figure 3B. **(G)** Visualization of most
962 representative genes from the integrative transcriptional regulatory networks.
963 Both RNA-seq and ATAC-seq data were integrated to uncover the transcriptional
964 regulatory networks with the paired expression and chromatin accessibility
965 (PECA) model³¹. The size of the node represents the out-degree, which is the
966 number of target gene. The color of the node represents the expression fold-
967 change. **(H)** Gene expression of selective TFs shown in the Figure 3G.

968

969 **Figure 4. Design of CRISPR-activation system for GATA4. (A)** Schematic of
970 the constitutive TRE-regulated dCas9-VP64 and GATA4 sgRNA constructs. **(B)**
971 GATA4 was overexpressed by adding doxycycline (dox) from day 6 in presence
972 of TKIs during cardiac differentiation of H1-hESCs. **(C)** Quantitative gene
973 expression analysis of tetracycline response element (TRE)-regulated dCas9-
974 VP64 cells transduced with GATA4 sgRNAs. Data are reported as means ±
975 standard error of the mean (SEM). The *p* values were calculated between cells
976 with and without adding dox. **(D)** Expression of GATA4 protein on in
977 differentiated CMs using Western blot analysis.

978

979 **Figure 5. Overexpression of GATA4 restored Ca²⁺-handling and reinstated**
980 **sarcomere structures in differentiated cardiomyocytes upon developmental**
981 **exposure to TKIs. (A-C)** Bar charts represent alternations of Ca²⁺-handling
982 properties in TKI-treated cardiomyocytes after induction of GATA4 expression,
983 including increases in beating rate and amplitude, and decreased in time to peak,
984 TD90/50, and decay tau. Data are reported as means ± standard error of the
985 mean (SEM). The *p* values were calculated between TKI-treated cells with and
986 without adding doxycycline (dox). **(D)** Representative Ca²⁺-handling traces from
987 DMSO and sunitinib-treated cardiomyocytes with and without GATA4 induction.
988 **(E)** Immunostaining of differentiated cardiomyocytes with α-actinin (green),
989 TNNT2 (red), and DAPI (blue). Disarrangement of sarcomere structures in TKI-
990 treated cardiomyocytes were reinstated after induction of GATA4 expression.

991

992 **Figure 6. Overexpression of GATA4 restored mitochondrial function and**
993 **change mitochondrial morphology in differentiated cardiomyocytes. (A-C)**

994 Overexpression of *GATA4* enhanced mitochondrial oxygen consumption rate
995 (OCR) in TKI-treated cardiomyocytes. The data were normalized by cell
996 numbers. **(D)** Enhanced mitochondrial respiration was also shown in control
997 group (0.1% DMSO) by induction of *GATA4* expression. H1-hESCs (wide type,
998 H1-WT) and H1-hESCs carrying CRISPR-a for *GATA4* showed similar OCR
999 without adding doxycycline. **(E-F)** Overexpression of *GATA4* increased ATP
1000 production (E) and mitochondrial membrane potential (F) in TKI-treated
1001 cardiomyocytes. The *p* values were calculated between TKI-treated cells with
1002 and without adding doxycycline (dox). **(G-J)** Developmental exposure to TKIs
1003 caused smaller branch sizes in differentiated cardiomyocytes (H-J) compared to
1004 control group (G). **(K-N)** Overexpression of *GATA4* increased the branch sizes in
1005 differentiated cardiomyocytes. Mitochondria in live cells were stained with
1006 MitoTracker dye (green). Nucleus were stained with DAPI (Blue). **(O)**
1007 Quantitative analysis of mitochondrial branch sizes using the Fiji software with a
1008 published method⁹⁶.

1009
1010 **Figure 7. GATA4 occupancy analysis by ChIP-seq experiments.** **(A)** Venn
1011 diagram represents numbers of genomic DNA-binding locations of *GATA4*
1012 between DMSO and sunitinib. **(B)** GO enrichment analysis of genes close to +/-
1013 3000 bps of *GATA4*-binding sites corresponding to the Venn diagram (A). **(C)**
1014 Both ATAC-seq and ChIP-seq of *GATA4* data showed higher signals at enhancer
1015 region of *PPARGC1A*. **(D)** Quantitative gene expression analysis of *PPARGC1A*.
1016 Data are reported as means \pm standard error of the mean (SEM). The dox
1017 represents doxycycline. **(E)** Visualization of long-range *PPARGC1A* promoter
1018 interactions in iPSC-derived cardiomyocytes confirms direct interaction between
1019 *GATA4*-binding site and *PPARGC1A* anchor region. Red loop specifies individual
1020 long-range interaction called between the *GATA4* binding site coordinates and
1021 the associated anchor used to capture *PPARGC1A* promoter interactions in
1022 cardiomyocytes⁴⁵.

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1040 **SUPPLEMENTAL FIGURES & TABLES**

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1042 **Supplemental Figure 1. No significant differences in efficiency of CM**
1043 **differentiation were observed upon exposure to NOAELs of drugs. (A)**

1044 Determination of the no-observed-adverse-effect-levels (NOAELs) of drugs used
1045 in this study. **(B)** Flow cytometry analysis differentiated cardiomyocytes of each
1046 group. The differentiated cardiomyocytes (day20) were labeled with TNNT2
1047 antibodies and used for the flow cytometry analysis. The data were collected
1048 from Exposure II, *i.e.*, the cells were exposure to TKI drugs till the differentiated
1049 CMs were collected for analyses.

1050

1051 **Supplemental Figure 2. Evaluation of Ca²⁺-handling of differentiated**
1052 **cardiomyocytes and expression of genes related to contractile. (A)** Bar

1053 charts represent developmental exposure to NOAELs of immunosuppressant
1054 (tacrolimus and mycophenolate) and thalidomide caused alternations of Ca²⁺-
1055 handling properties, including decreases in beating rate and amplitude, and
1056 increased in time to peak, TD90/50, and decay tau. The data were collected from
1057 Exposure II. The *p* values were calculated by nonparametric T-test between
1058 control and TKI-treated groups. NA, no significance was observed (*p*>0.05). **(B)**
1059 Gene expression of genes related to contraction of cardiomyocyte. The color of
1060 the heatmap represents log₂-transformed fold changes, which was calculated by
1061 treatment vs. DMSO using the normalized counts by trimmed mean of M-values
1062 (TMM) normalization method.

1063

1064 **Supplemental Figure 3. Motif analysis in TKI-treated cells and control cells**
1065 **from ATAC-seq data. (A)** Enriched de novo motifs for MEF2s family were

1066 discovered using MEME-chip based on down-regulated chromatin accessibility in
1067 TKI-treated cells. **(B)** Peak signals (+/- 1000 bps) arounds submits of the motifs
1068 for GATA4, TEAD1, and TEAD3 in TKI-treated cells compared with DMSO-
1069 treated cells. Binding intensities are shown as sequencing depth normalized tag
1070 count.

1071

1072 **Supplemental Figure 4. Gene expression of GATA4 during cardiomyocyte**
1073 **differentiation. (A)** Data represent normalized counts collected from three time

1074 points of the RNA-seq data: day 0, day 6, and day 20. **(B)** Protein expression
1075 analysis of GATA4 in two sgRNA designs. Two sgRNA were designed for
1076 targeting upstream of TSS of GATA4. Cells from intermediate differentiation
1077 stages were collected for protein analysis using Western blot

1078

1079 **Supplemental Figure 5. Changes of mitochondrial DNA copies during**
1080 **cardiomyocyte differentiation upon TKI exposure. (A)** Dynamic mitochondrial

1081 DNA (mtDNA) copies during cardiomyocyte differentiation in control (0.1%
1082 DMSO). Data were collected from four time points: day 6, day 9, day 23, day 26.
1083 **(B-C)** TKI-exposure decreased mtDNA copies compared to control. However, no
1084 significant differences were observed. NA, *p*>0.05. The *p* values were calculated
1085 by nonparametric T-test between control and TKI-treated groups. **(D)**

1086 Overexpression of GATA4 by adding doxycycline (dox) increased mtDNA copies
1087 in TKI-treated cells. Data were collected from cardiomyocytes of day26. The *p*
1088 values were calculated between TKI-treated cells with and without
1089 overexpressing GATA4. **(E)** Protein expression analysis of PGC-1 α in
1090 cardiomyocytes.

1091

1092 **Supplemental Figure 6. Maternal exposure to sunitinib induced moderate**
1093 **pathology in offspring. (A)** Experimental design of maternal exposure using
1094 mouse model. **(B)** Example of death of fetus due to maternal exposure to
1095 sunitinib. **(C)** Moderate CHD-like histopathologic outcomes were observed in
1096 fetal mice (E14.5). TM, thinner myocardium; VSD, ventricular septal defect. Scale
1097 bars: 200 μ m. **(D)** Bioaccumulation of sunitinib was found in the blood of both
1098 female mouse and newborns (P0). Only one adult female mouse was used.

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1102 **Supplemental Table 1. Gene lists of each module from WGCNA.**

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1104 **Supplemental Table 2. Summary of each module from WGCNA.**

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1106 **Supplemental Table 3. ChIP-seq analysis of GATA4 occupancy in**
1107 **cardiomyocytes from both control and sunitinib-treated groups.**

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1152 **AUTHOR CONTRIBUTIONS**

1153
1154 Q.L. conceived and designed the experiments under the supervision of M.P.S..
1155 Q.L. performed most of experiments and data analysis. H.W performed Ca^{2+}
1156 handling analysis and microscopy. Q.J.L. and J.L. performed the mice studies.
1157 C.J. and Z.D. performed network analysis. M.T.Z. carried out the flow cytometry
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1172 REFERENCES

- 1173 1 Klimas, J. in *Cardiomyopathies – From Basic Research to Clinical*
1174 *Management* (ed J Veselka) Ch. 26, 581-620 (InTech, 2012).
- 1175 2 Bjornard, K., Riehle-Colarusso, T., Gilboa, S. & Correa, A. Patterns in the
1176 prevalence of congenital heart defects, metropolitan Atlanta, 1978 to
1177 2005. *Birth Defects Res A Clin Mol Teratol.* **97**, 87-94 (2013).
- 1178 3 Hoffman, J. & Kaplan, S. The incidence of congenital heart disease. *J Am*
1179 *Coll Cardiol.* **39**, 1890–1900 (2002).
- 1180 4 Jenkins, K. *et al.* Noninherited risk factors and congenital cardiovascular
1181 defects: current knowledge: a scientific statement from the American
1182 Heart Association Council on Cardiovascular Disease in the Young:
1183 endorsed by the American Academy of Pediatrics. *Circulation* **115**, 2995-
1184 3014 (2007).
- 1185 5 Van Vliet, P., Wu, S., Zaffran, S. & Pucéat, M. Early cardiac development:
1186 a view from stem cells to embryos. *Cardiovasc Res.* **96**, 352-362 (2012).
- 1187 6 Zhu, Z. & Huangfu, D. Human pluripotent stem cells: an emerging model
1188 in developmental biology. *Development* **140**, 705-717 (2013).
- 1189 7 Liu, Q. *et al.* Disruption of mesoderm formation during cardiac
1190 differentiation due to developmental exposure to 13-cis-retinoic acid. *Sci*
1191 *Rep.* **8**, 12960 (2018).
- 1192 8 Abruzzese, E., Trawinska, M., Perrotti, A. & De Fabritiis, P. Tyrosine
1193 kinase inhibitors and pregnancy. *Mediterr J Hematol Infect Dis.* **6**,
1194 e2014028 (2014).
- 1195 9 Pye, S. *et al.* The effects of imatinib on pregnancy outcome. *Blood* **111**,
1196 5505-5508 (2008).
- 1197 10 Scheffel, R. *et al.* Toxic cardiomyopathy leading to fatal acute cardiac
1198 failure related to vandetanib: a case report with histopathological analysis.
1199 *Eur J Endocrinol.* **168**, K51-54 (2013).
- 1200 11 Jain, A. *et al.* Pregnancy after liver transplantation with tacrolimus
1201 immunosuppression: a single center's experience update at 13 years. *.*
1202 *Transplantation.* **76**, 827-832 (2003).
- 1203 12 Wenners, A. *et al.* Fetal hypoplastic left heart syndrome and maternal liver
1204 transplantation for Wilson's disease: a case report. *. J Med Case Rep* **7**,
1205 276 (2013).
- 1206 13 Kim, J. & Scialli, A. Thalidomide: the tragedy of birth defects and the
1207 effective treatment of disease. *Toxicol Sci.* **122**, 1-6 (2011).
- 1208 14 Miller, M. & Strömmland, K. Teratogen update: thalidomide: a review, with a
1209 focus on ocular findings and new potential uses. *Teratology* **60**, 306-321
1210 (1999).
- 1211 15 Jovelet, C. *et al.* Variation in transplacental transfer of tyrosine kinase
1212 inhibitors in the human perfused cotyledon model. *Ann Oncol.* **26**, 1500-
1213 1504 (2015).
- 1214 16 Moran, B., Yano, H., Al Zahir, N. & Farquharson, M. Conflicting priorities in
1215 surgical intervention for cancer in pregnancy. *Lancet Oncol* **8**, 536-544
1216 (2007).

- 1217 17 Pentheroudakis, G. & Pavlidis, N. Cancer and pregnancy: poena magna,
1218 not anymore. *Eur J Cancer*. **42**, 126-140 (2006).
- 1219 18 Apperley, J. Issues of imatinib and pregnancy outcome. *J Natl Compr*
1220 *Canc Netw* **7**, 1050-1058 (2009).
- 1221 19 Webb, M. & D, J. Imatinib use in pregnancy. *Turk J Haematol*. **29**, 405-
1222 408. (2012).
- 1223 20 Burridge, P. *et al*. Chemically defined generation of human
1224 cardiomyocytes. *Nat Methods*. **11**, 855-860 (2014).
- 1225 21 Andriamanana, I., Gana, I., Duretz, B. & Hulin, A. Simultaneous analysis
1226 of anticancer agents bortezomib, imatinib, nilotinib, dasatinib, erlotinib,
1227 lapatinib, sorafenib, sunitinib and vandetanib in human plasma using
1228 LC/MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **926**, 83-91
1229 (2013).
- 1230 22 Demetri, G. *et al*. Efficacy and safety of sunitinib in patients with advanced
1231 gastrointestinal stromal tumour after failure of imatinib: a randomised
1232 controlled trial. *Lancet*. 2006 Oct 14;368(9544): **362**, 1329-1338 (2006).
- 1233 23 Gschwind, H. *et al*. Metabolism and disposition of imatinib mesylate in
1234 healthy volunteers. *Drug Metab Dispos*. **33**, 1503-1512 (2005).
- 1235 24 Motzer, R. *et al*. Sunitinib versus interferon alfa in metastatic renal-cell
1236 carcinoma. *N Engl J Med*. **352**, 115-124 (2007).
- 1237 25 Ton, G., Banaszynski, M. & Kolesar, J. Vandetanib: a novel targeted
1238 therapy for the treatment of metastatic or locally advanced medullary
1239 thyroid cancer. *Am J Health Syst Pharm* **70**, 849-855 (2013).
- 1240 26 Ellen Kreipke, R., Wang, Y., Miklas, J., Mathieu, J. & Ruohola-Baker, H.
1241 Metabolic remodeling in early development and cardiomyocyte maturation.
1242 *Semin Cell Dev Biol* **52**, 84-92 (2016).
- 1243 27 Lopaschuk, G. & Jaswal, J. Energy metabolic phenotype of the
1244 cardiomyocyte during development, differentiation, and postnatal
1245 maturation. *J Cardiovasc Pharmacol* **56**, 130-140 (2010).
- 1246 28 Piquereau, J. & Ventura-Clapier, R. Maturation of Cardiac Energy
1247 Metabolism During Perinatal Development. *Front Physiol.*,
1248 10.3389/fphys.2018.00959 (2018).
- 1249 29 Buenrostro, J., Giresi, P., Zaba, L., Chang, H. & Greenleaf, W.
1250 Transposition of native chromatin for fast and sensitive epigenomic
1251 profiling of open chromatin, DNA-binding proteins and nucleosome
1252 position. *Nat Methods*. **10**, 1213-1218 (2013).
- 1253 30 Buenrostro, J., Wu, B., Chang, H. & Greenleaf, W. ATAC-seq: A Method
1254 for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol*,
1255 21.29.21-29 (2015).
- 1256 31 Duren, Z., Chen, X., Jiang, R., Wang, Y. & Wong, W. Modeling gene
1257 regulation from paired expression and chromatin accessibility data. *Proc*
1258 *Natl Acad Sci U S A* **114**, E4914-E4923 (2017).
- 1259 32 Duren, Z., Chen, X., Xin, J., Wang, Y. & Wong, W. Time course regulatory
1260 analysis based on paired expression and chromatin accessibility data.
1261 *Genome Res pii: gr.257063.119*, doi: 10.1101/gr.257063.257119. (2020).

- 1262 33 Fan, W. & Evans, R. PPARs and ERRs: molecular mediators of
1263 mitochondrial metabolism. *Curr Opin Cell Biol* **33**, 49-54 (2015).
- 1264 34 LeBleu, V. *et al.* PGC-1 α mediates mitochondrial biogenesis and oxidative
1265 phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol*, 1-15
1266 (2014).
- 1267 35 Wu, Z. *et al.* Mechanisms controlling mitochondrial biogenesis and
1268 respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-124
1269 (1999).
- 1270 36 Horb, M. & Thomsen, G. Tbx5 is essential for heart development.
1271 *Development* **126**, 1739-1751 (1999).
- 1272 37 Chung, S. *et al.* Mitochondrial oxidative metabolism is required for the
1273 cardiac differentiation of stem cells. *Nat Clin Pract Cardiovasc Med.* **4**, 60-
1274 67 (2007).
- 1275 38 Dorn, G., Vega, R. & Kelly, D. Mitochondrial biogenesis and dynamics in
1276 the developing and diseased heart. *Genes Dev.* **29**, 1981-1991 (2015).
- 1277 39 Hom, J. *et al.* The permeability transition pore controls cardiac
1278 mitochondrial maturation and myocyte differentiation. *Dev Cell* **21**, 469-
1279 478 (2011).
- 1280 40 He, A. *et al.* Dynamic GATA4 enhancers shape the chromatin landscape
1281 central to heart development and disease. *Nat Commun.* **5**, doi:
1282 10.1038/ncomms5907 (2014).
- 1283 41 Kearns, N. *et al.* Cas9 effector-mediated regulation of transcription and
1284 differentiation in human pluripotent stem cells. *Development* **141**, 219-223
1285 (2014).
- 1286 42 Sanjana, N., Shalem, O. & Zhang, F. Improved vectors and genome-wide
1287 libraries for CRISPR screening. *Nat Methods* **11**, 783-784 (2014).
- 1288 43 Zhao, Q., Sun, Q., Zhou, L., Liu, K. & Jiao, K. Complex Regulation of
1289 Mitochondrial Function During Cardiac Development. *J Am Heart Assoc* **8**,
1290 e012731 (2019).
- 1291 44 Irrcher, I., Ljubicic, V., Kirwan, A. & Hood, D. AMP-activated protein
1292 kinase-regulated activation of the PGC-1 α promoter in skeletal muscle
1293 cells. *PLoS One* **3**, e3614 (2008).
- 1294 45 Montefiori, L. *et al.* A promoter interaction map for cardiovascular disease
1295 genetics. *Elife pii: e35788.*, doi: 10.7554/eLife.35788. (2018).
- 1296 46 Nicoll, R. Environmental Contaminants and Congenital Heart Defects: A
1297 Re-Evaluation of the Evidence. *Int J Environ Res Public Health.* **15**, 2096
1298 (2018).
- 1299 47 Liu, Z. *et al.* Maternal lead exposure and risk of congenital heart defects
1300 occurrence in offspring. *Reprod Toxicol.* **51**, 1-6 (2014).
- 1301 48 Jin, X. *et al.* Maternal exposure to arsenic and cadmium and the risk of
1302 congenital heart defects in offspring. *Reprod Toxicol.* **59**, 109-116 (2016).
- 1303 49 Kalliora, C. *et al.* Association of pesticide exposure with human congenital
1304 abnormalities. *Toxicol Appl Pharmacol.* **346**, 58-75 (2018).
- 1305 50 Rocheleau, C. *et al.* Maternal occupational pesticide exposure and risk of
1306 congenital heart defects in the National Birth Defects Prevention Study.
1307 *Birth Defects Res A Clin Mol Teratol.* **103**, 823-833 (2015).

- 1308 51 Autret-Leca, E. *et al.* Isotretinoin exposure during pregnancy: assessment
1309 of spontaneous reports in France. *Drug Saf.* **33**, 659-665 (2010).
- 1310 52 Dai, W., LaBraico, J. & Stern, R. Epidemiology of isotretinoin exposure
1311 during pregnancy. *J Am Acad Dermatol.* **26**, 599–606 (1992).
- 1312 53 Lammer, E. *et al.* Retinoic acid embryopathy. *N Engl J Med.* **313**, 837-841
1313 (1985).
- 1314 54 Hall, P., Harshman, L., Srinivas, S. & Witteles, R. The frequency and
1315 severity of cardiovascular toxicity from targeted therapy in advanced renal
1316 cell carcinoma patients. *JACC: Heart Failure* **1**, 72-78 (2013).
- 1317 55 Lenihan, D. & Kowey, P. Overview and management of cardiac adverse
1318 events associated with tyrosine kinase inhibitors. *Oncologist.* **18**, 900-908
1319 (2013).
- 1320 56 Xu, Z., Cang, S., Yang, T. & Liu, D. Cardiotoxicity of tyrosine kinase
1321 inhibitors in chronic myelogenous leukemia therapy. *Hematol Rev.* **1**, e4
1322 (2009).
- 1323 57 Chen, M., Kerkelä, R. & Force, T. Mechanisms of cardiac dysfunction
1324 associated with tyrosine kinase inhibitor cancer therapeutics. *Circulation*
1325 **118**, 84-95 (2008).
- 1326 58 Rees, M. *et al.* A PKM2 signature in the failing heart. *Biochem Biophys*
1327 *Res Commun* **459**, 430–436 (2015).
- 1328 59 Wang, X. *et al.* Germline mutations in ABL1 cause an autosomal dominant
1329 syndrome characterized by congenital heart defects and skeletal
1330 malformations. *Nat Genet.* **49**, 613-617 (2017).
- 1331 60 Tang, X. *et al.* PDGFRA gene, maternal binge drinking and obstructive
1332 heart defects. *Sci Rep.* **8**, 11083 (2018).
- 1333 61 Richarte, A., Mead, H. & Tallquist, M. Cooperation between the PDGF
1334 receptors in cardiac neural crest cell migration. *Developmental biology*
1335 **306** (2007).
- 1336 62 Yilbas, A. *et al.* Activation of GATA4 gene expression at the early stage of
1337 cardiac specification. *Front. Chem* **2**,
1338 <https://doi.org/10.3389/fchem.2014.00012> (2014).
- 1339 63 Grépin, C., Nemer, G. & Nemer, M. Enhanced cardiogenesis in embryonic
1340 stem cells overexpressing the GATA-4 transcription factor. *Development.*
1341 **124**, 2387-2395 (1997).
- 1342 64 Liang, Q. *et al.* The transcription factors GATA4 and GATA6 regulate
1343 cardiomyocyte hypertrophy in vitro and in vivo. *J Biol Chem.* **276**, 30245-
1344 30253 (2001).
- 1345 65 Malek Mohammadi, M. *et al.* The transcription factor GATA4 promotes
1346 myocardial regeneration in neonatal mice. *EMBO Mol Med* **9**, 265-279
1347 (2017).
- 1348 66 Heineke, J. *et al.* Cardiomyocyte GATA4 functions as a stress-responsive
1349 regulator of angiogenesis in the murine heart. *J Clin Invest.* **117**, 3198-
1350 3210 (2007).
- 1351 67 Aries, A., Paradis, P., Lefebvre, C., Schwartz, R. & Nemer, M. Essential
1352 role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proc Natl*
1353 *Acad Sci U S A.* **101**, 6975-6980 (2004).

- 1354 68 Kim, Y. *et al.* Anthracycline-induced suppression of GATA-4 transcription
1355 factor: implication in the regulation of cardiac myocyte apoptosis. *Mol*
1356 *Pharmacol* **63**, 368-377 (2003).
- 1357 69 Maharsy, W., Aries, A., Mansour, O., Komati, H. & Nemer, M. Ageing is a
1358 risk factor in imatinib mesylate cardiotoxicity. *Eur J Heart Fail* **16**, 367-376
1359 (2014).
- 1360 70 Beutner, G., Eliseev, R. & Porter, G. J. Initiation of electron transport chain
1361 activity in the embryonic heart coincides with the activation of
1362 mitochondrial complex 1 and the formation of supercomplexes. *PLoS One*
1363 **9**, e113330 (2014).
- 1364 71 Will, Y. *et al.* Effect of the multitargeted tyrosine kinase inhibitors imatinib,
1365 dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat
1366 heart mitochondria and H9c2 cells. *Toxicol Sci* **106**, 153-161 (2008).
- 1367 72 Janssen, L. *et al.* Skeletal muscle toxicity associated with tyrosine kinase
1368 inhibitor therapy in patients with chronic myeloid leukemia. *Leukemia* **33**,
1369 2116-2120 (2019).
- 1370 73 Paech, F., Bouitbir, J. & Krähenbühl, S. Hepatocellular Toxicity Associated
1371 with Tyrosine Kinase Inhibitors: Mitochondrial Damage and Inhibition of
1372 Glycolysis. *Front Pharmacol.* **8**, doi: 10.3389/fphar.2017.00367 (2017).
- 1373 74 Kasahara, A., Cipolat, S., Chen, Y., Dorn, G. & Scorrano, L. Mitochondrial
1374 fusion directs cardiomyocyte differentiation via calcineurin and Notch
1375 signaling. *Science* **342**, 734-737 (2013).
- 1376 75 Creemers, E., Sutherland, L., McAnally, J., Richardson, J. & Olson, E.
1377 Myocardin is a direct transcriptional target of Mef2, Tead and Foxo
1378 proteins during cardiovascular development. *Development.* 2006
1379 *Nov*;133(21): *Epub 2006 Oct 4.* **133**, 4245-4256 (2006).
- 1380 76 Liu, R. *et al.* Tead1 is required for perinatal cardiomyocyte proliferation.
1381 *PLoS One.* 2019 *Feb 27*;14(2): **14**, e0212017 (2019).
- 1382 77 Desjardins, C. & Naya, F. The Function of the MEF2 Family of
1383 Transcription Factors in Cardiac Development, Cardiogenomics, and
1384 Direct Reprogramming. *J Cardiovasc Dev Dis* **3**, pii: 26. (2016).
- 1385 78 Czubyrt, M., McAnally, J., Fishman, G. & Olson, E. Regulation of
1386 peroxisome proliferator-activated receptor gamma coactivator 1 alpha
1387 (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. *Proc*
1388 *Natl Acad Sci U S A.* **100**, 1711-1716 (2003).
- 1389 79 Mammoto, A., Muyleart, M., Kadlec, A., Gutterman, D. & Mammoto, T.
1390 YAP1-TEAD1 signaling controls angiogenesis and mitochondrial
1391 biogenesis through PGC1 α . *Microvasc Res* **119**, 73-83 (2018).
- 1392 80 Kumar, R. *et al.* Regulation of energy metabolism during early mammalian
1393 development: TEAD4 controls mitochondrial transcription. *Development*
1394 **145**, pii: dev162644 (2018).
- 1395 81 He, A., Kong, S., Ma, Q. & Pu, W. Co-occupancy by multiple cardiac
1396 transcription factors identifies transcriptional enhancers active in heart.
1397 *Proc Natl Acad Sci U S A.* **108**, 5632-5637 (2011).

- 1398 82 Morin, S., Charron, F., Robitaille, L. & Nemer, M. GATA-dependent
1399 recruitment of MEF2 proteins to target promoters. *EMBO J.* **19**, 2046–
1400 2055 (2000).
- 1401 83 Sharma, A. *et al.* Derivation of highly purified cardiomyocytes from human
1402 induced pluripotent stem cells using small molecule-modulated
1403 differentiation and subsequent glucose starvation. *J Vis Exp* **97** (2015).
- 1404 84 Wu, H. *et al.* Epigenetic Regulation of Phosphodiesterases 2A and 3A
1405 Underlies Compromised β -Adrenergic Signaling in an iPSC Model of
1406 Dilated Cardiomyopathy. *Cell Stem Cell.* **17**, 89-100 (2015).
- 1407 85 Anders, S., Pyl, P. & Huber, W. HTSeq--a Python framework to work with
1408 high-throughput sequencing data. *Bioinformatics.* **31**, 166-169 (2015).
- 1409 86 Robinson, M. & Oshlack, A. A scaling normalization method for differential
1410 expression analysis of RNA-seq data. *Genome Biology* **11**, R25
1411 (2010).
- 1412 87 Consortium., E. P. An integrated encyclopedia of DNA elements in the
1413 human genome. *Nature* **489**, 57-74 (2012).
- 1414 88 Ross-Innes, C. *et al.* Differential oestrogen receptor binding is associated
1415 with clinical outcome in breast cancer. *Nature* **481**, 389-393 (2012).
- 1416 89 Stark, R. & Brown, G. DiffBind: differential binding analysis of ChIP-Seq
1417 peak data. *Bioconductor*,
1418 <http://bioconductor.org/packages/release/bioc/vignettes/DiffBind/inst/doc/DiffBind.pdf> (2011).
- 1419 90 Machanick, P. & Bailey, T. MEME-ChIP: motif analysis of large DNA
1420 datasets. *Bioinformatics.* **27**, 1696-1697 (2011).
- 1421 91 Grant, C., Bailey, T. & Noble, W. FIMO: Scanning for occurrences of a
1422 given motif. *Bioinformatics* **27**, 1017–1018 (2011).
- 1423 92 Heinz, S. *et al.* Simple Combinations of Lineage-Determining Transcription
1424 Factors Prime cis-Regulatory Elements Required for Macrophage and B
1425 Cell Identities. *Mol Cell* **38**, 576-589 (2010).
- 1426 93 Shen, L., Shao, N., Liu, X. & Nestler, E. ngs.plot: Quick mining and
1427 visualization of next-generation sequencing data by integrating genomic
1428 databases. *BMC Genomics* **15**, 284 (2014).
- 1429 94 Phanstiel, D., Boyle, A., Araya, C. & Snyder, M. Sushi.R: flexible,
1430 quantitative and integrative genomic visualizations for publication-quality
1431 multi-panel figures. *Bioinformatics* **30**, 2808-2810 (2014).
- 1432 95 Langfelder, P. & Horvath, S. WGCNA: an R package for weighted
1433 correlation network analysis. *BMC Bioinformatics* **9**, doi: 10.1186/1471-
1434 2105-1189-1559 (2008).
- 1435 96 Valente, A., Maddalena, L., Robb, E., Moradi, F. & Stuart, J. A simple
1436 ImageJ macro tool for analyzing mitochondrial network morphology in
1437 mammalian cell culture. *Acta Histochem* **119**, 315-326 (2017).
- 1438 97 Pfaffl, M. A new mathematical model for relative quantification in real-time
1439 RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
- 1440 98 Marciano, D. & Snyder, M. Personalized Metabolomics. *Methods in*
1441 *Molecular Biology (Clifton, N.J.)* **1978**, 447-456 (2018).

1443

Figure 1

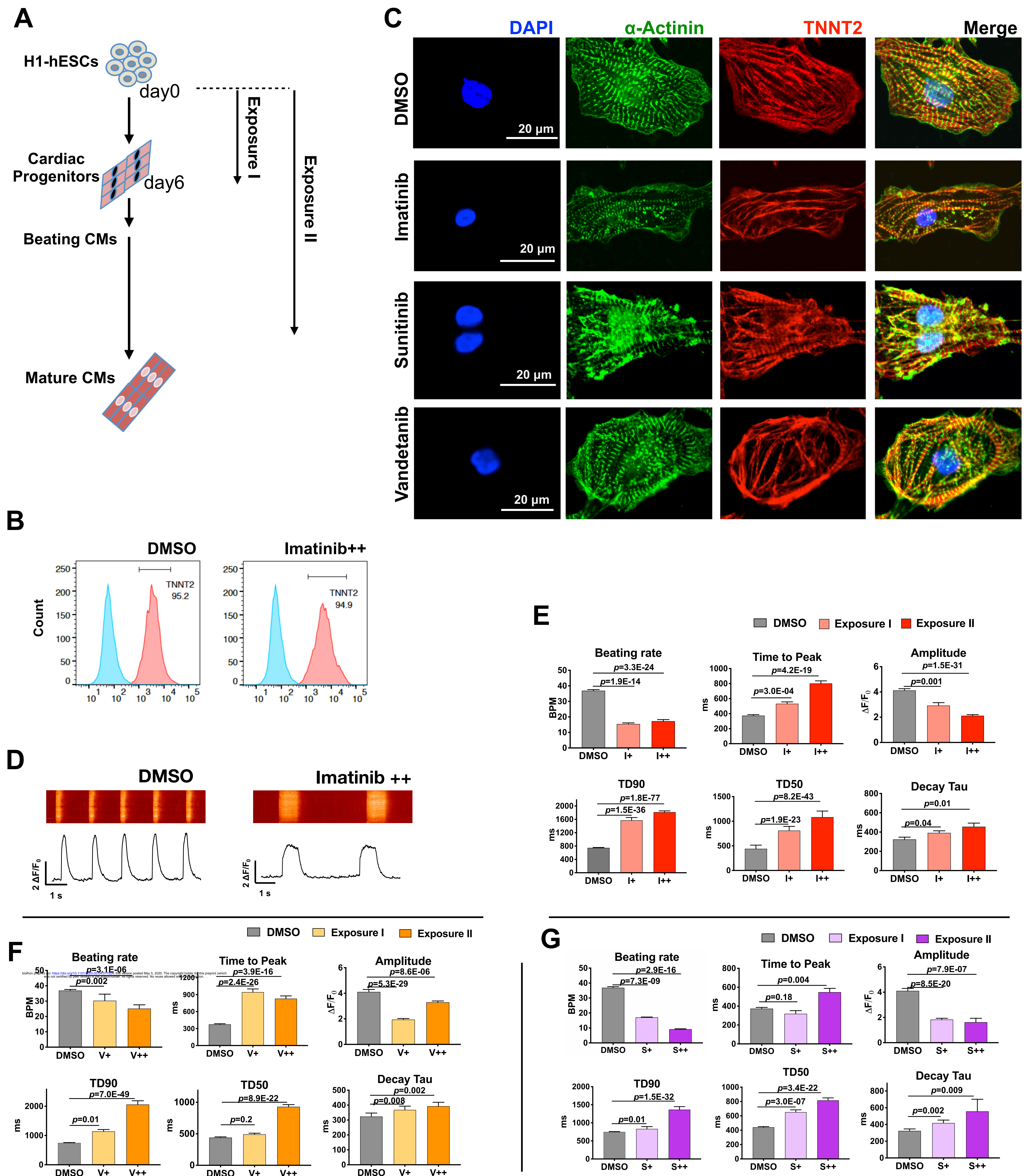


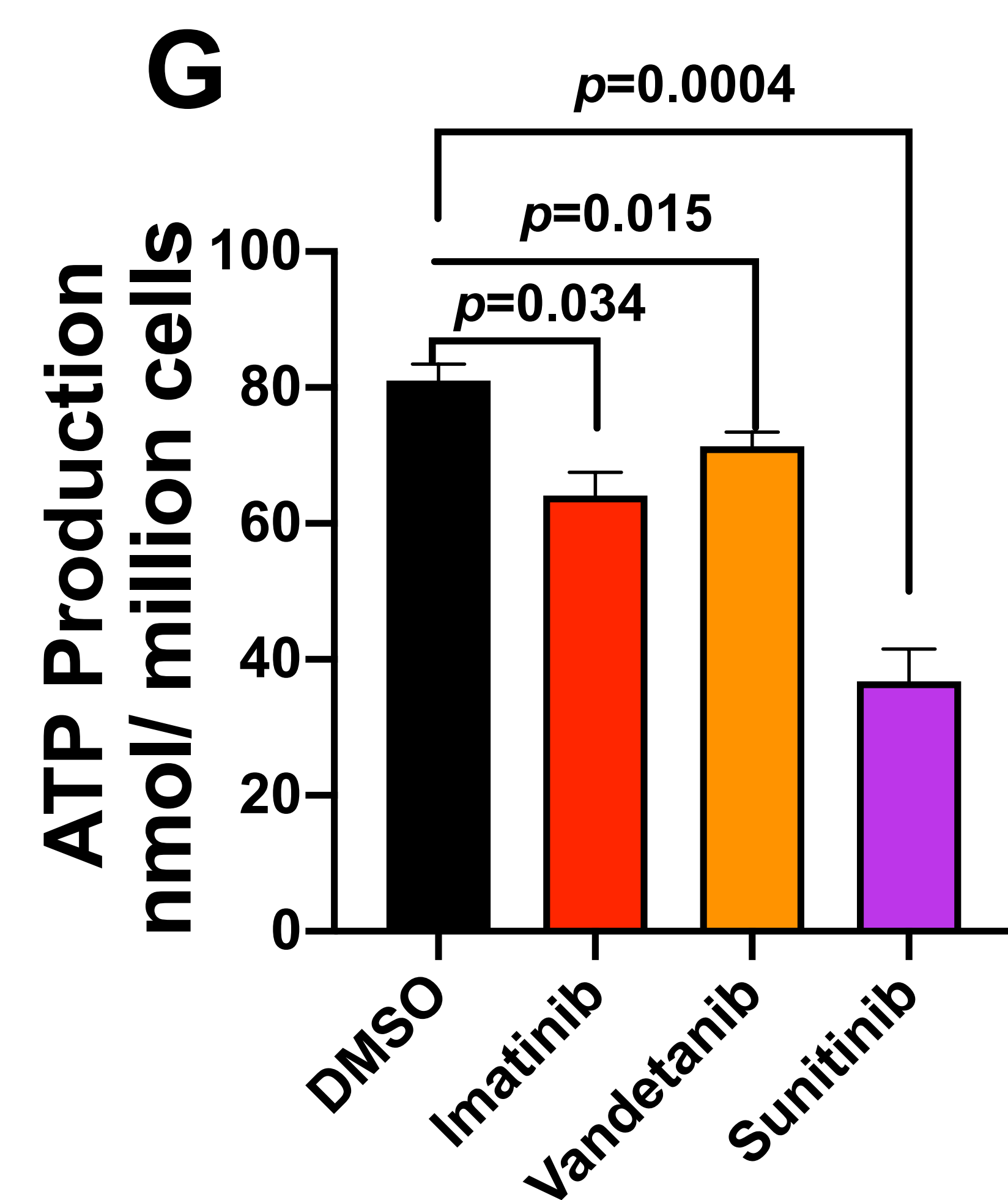
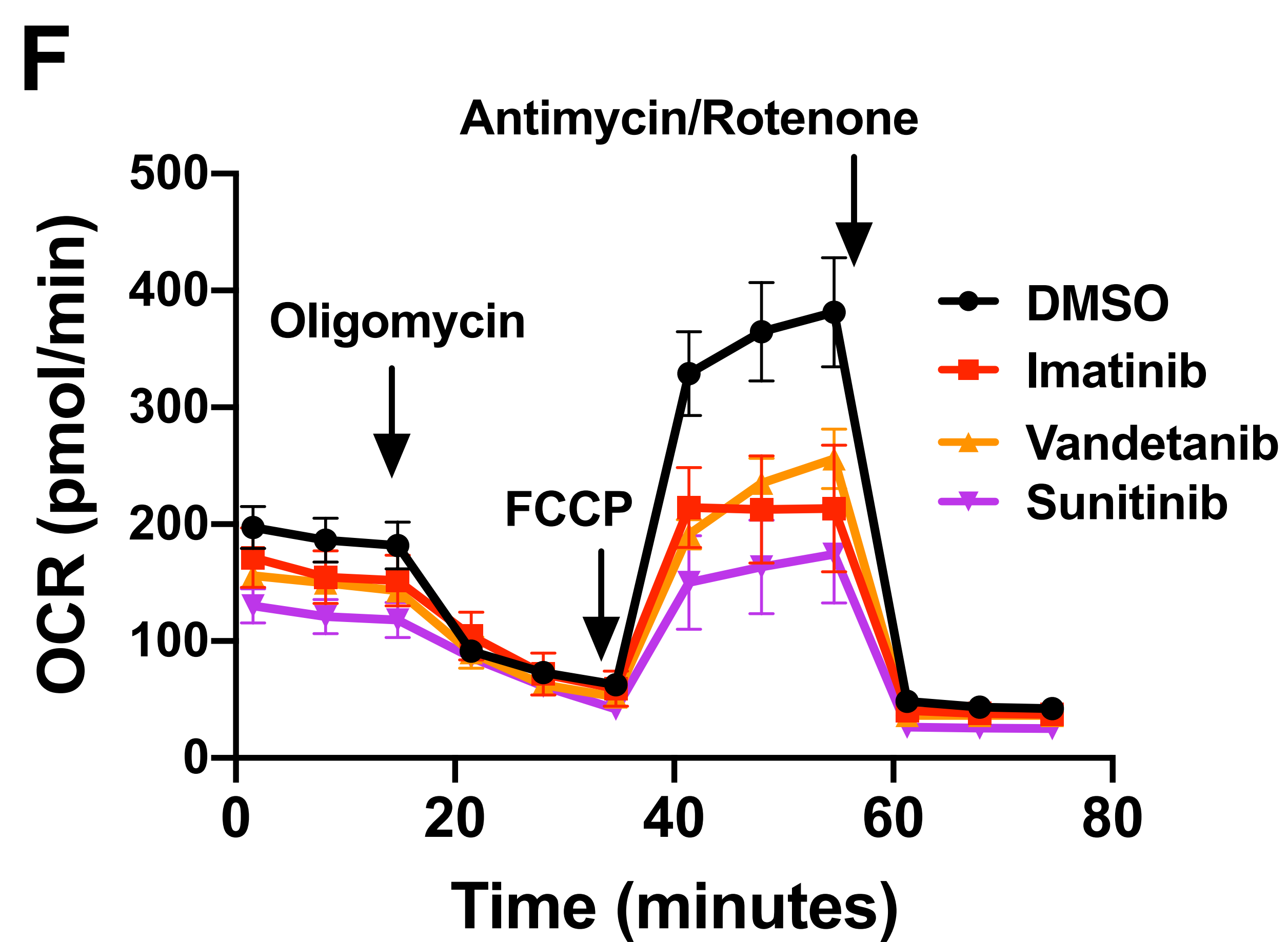
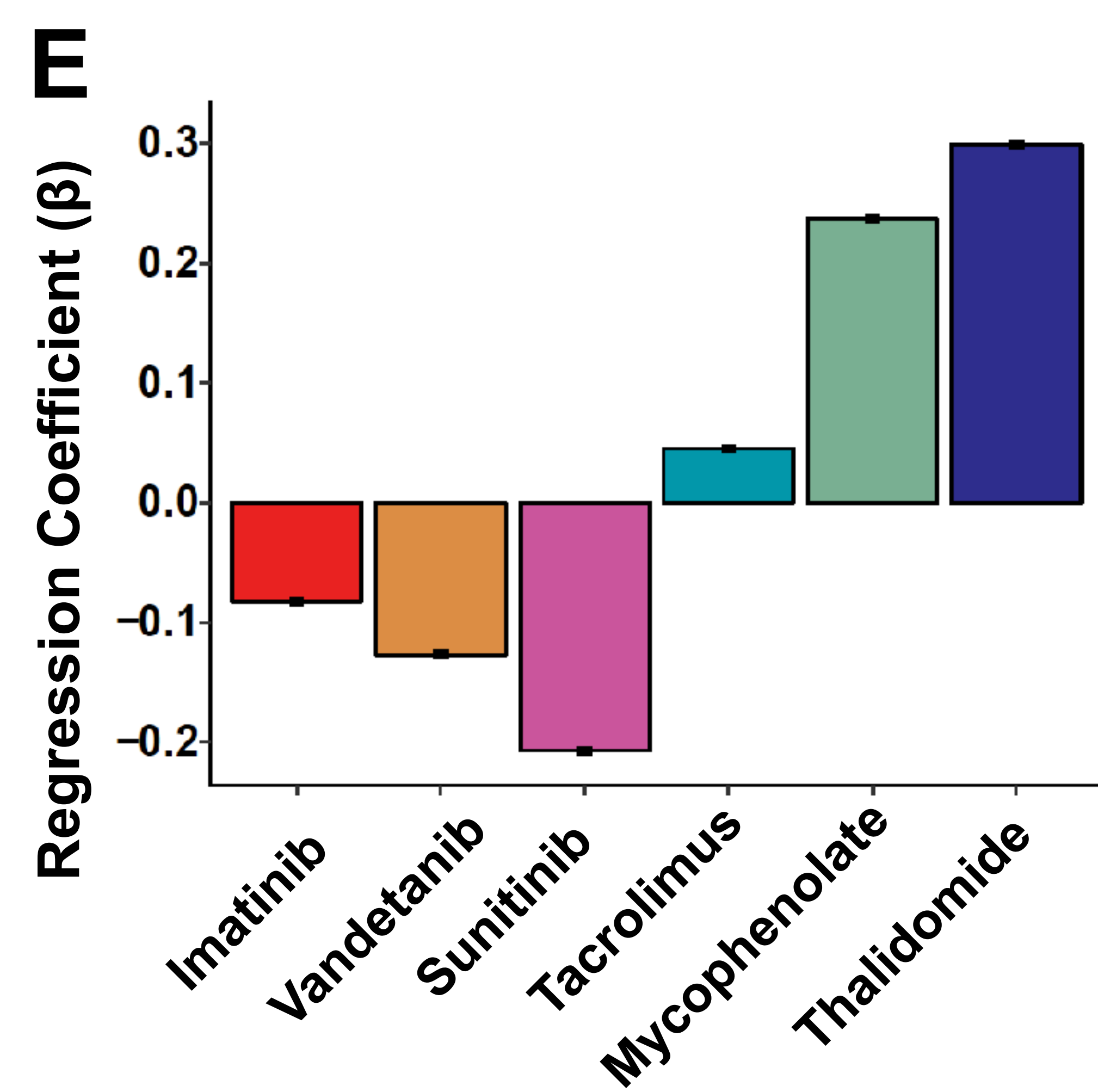
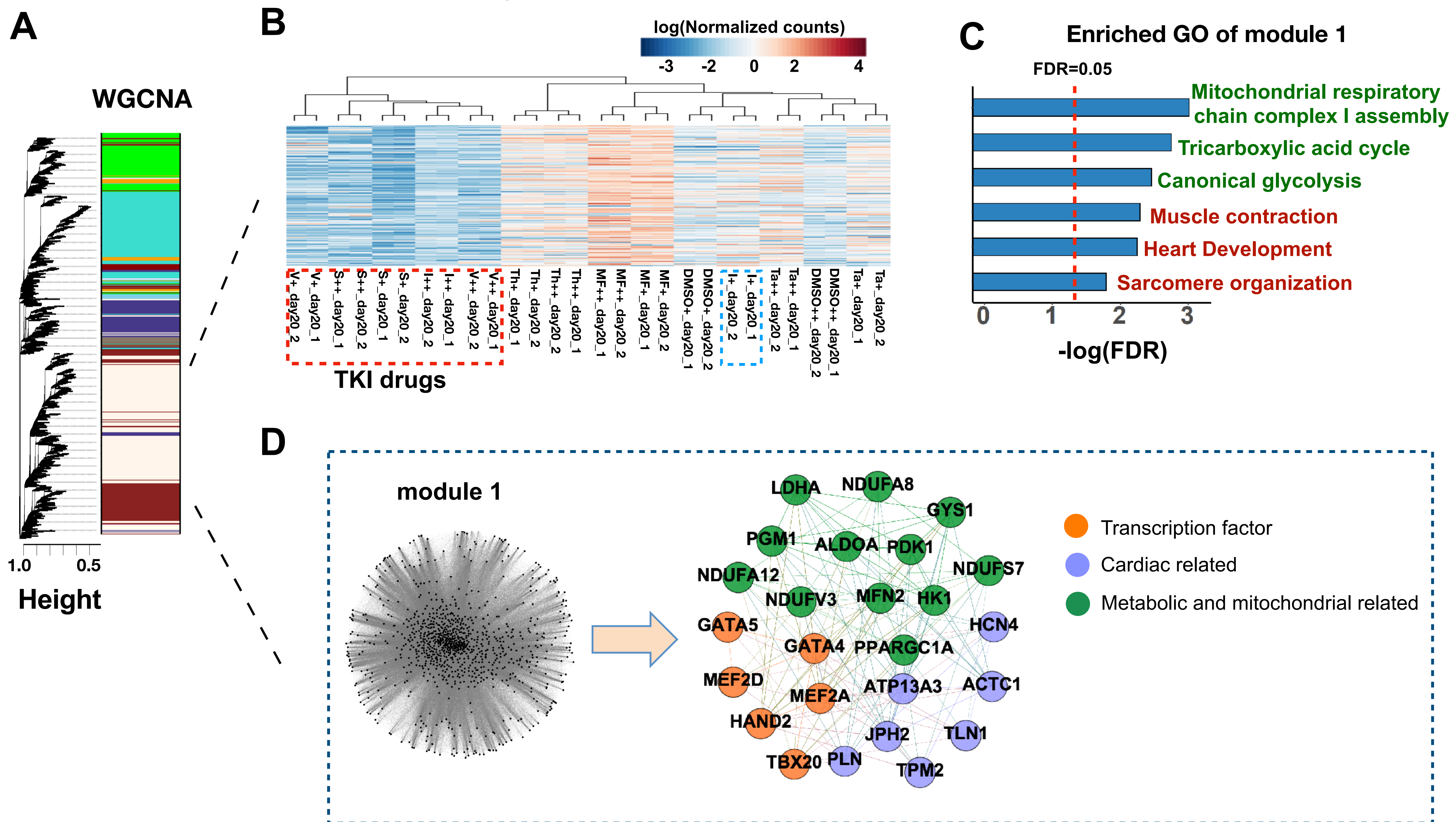
Figure 2**Gene expression in module 1**

Figure 3

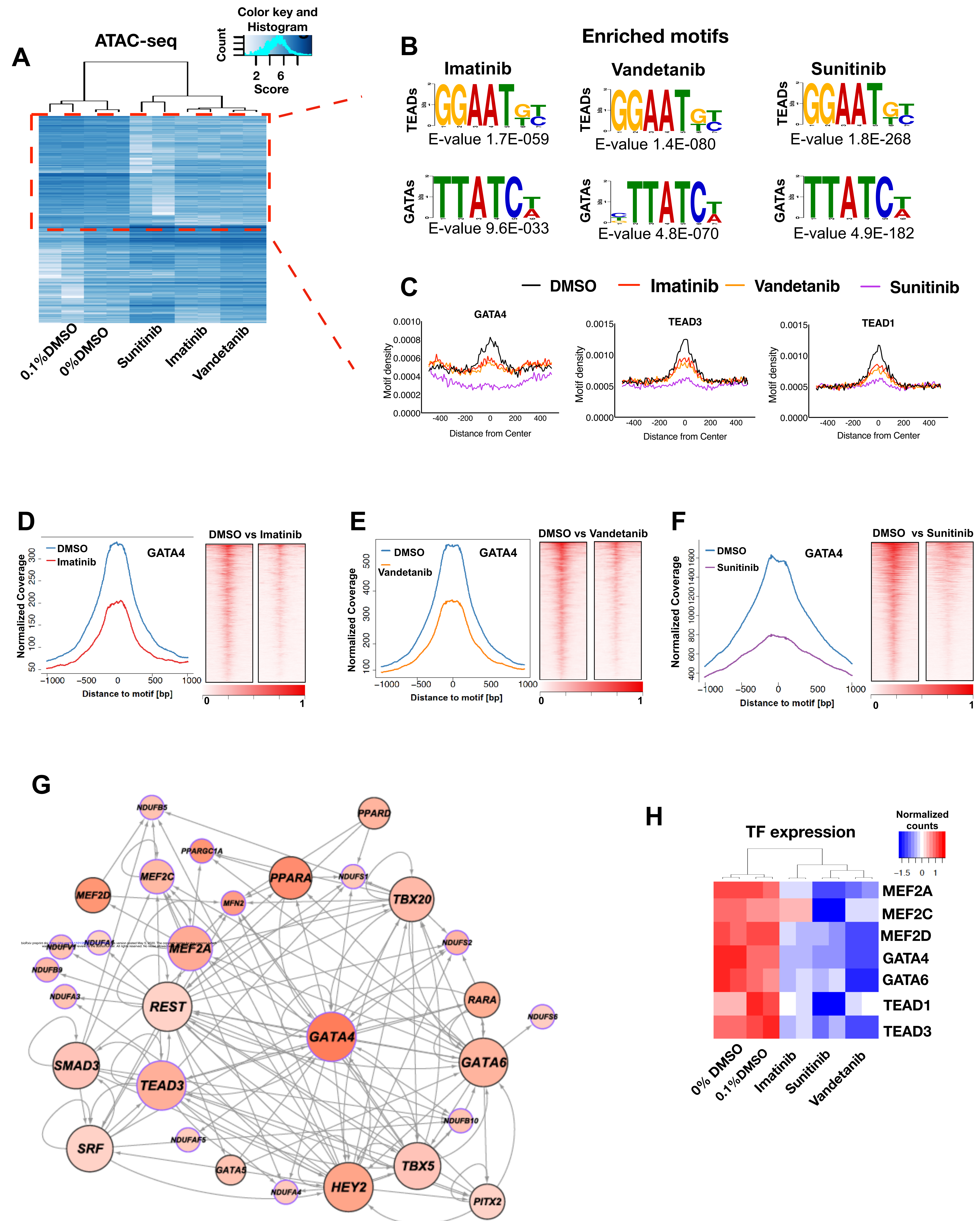


Figure 5

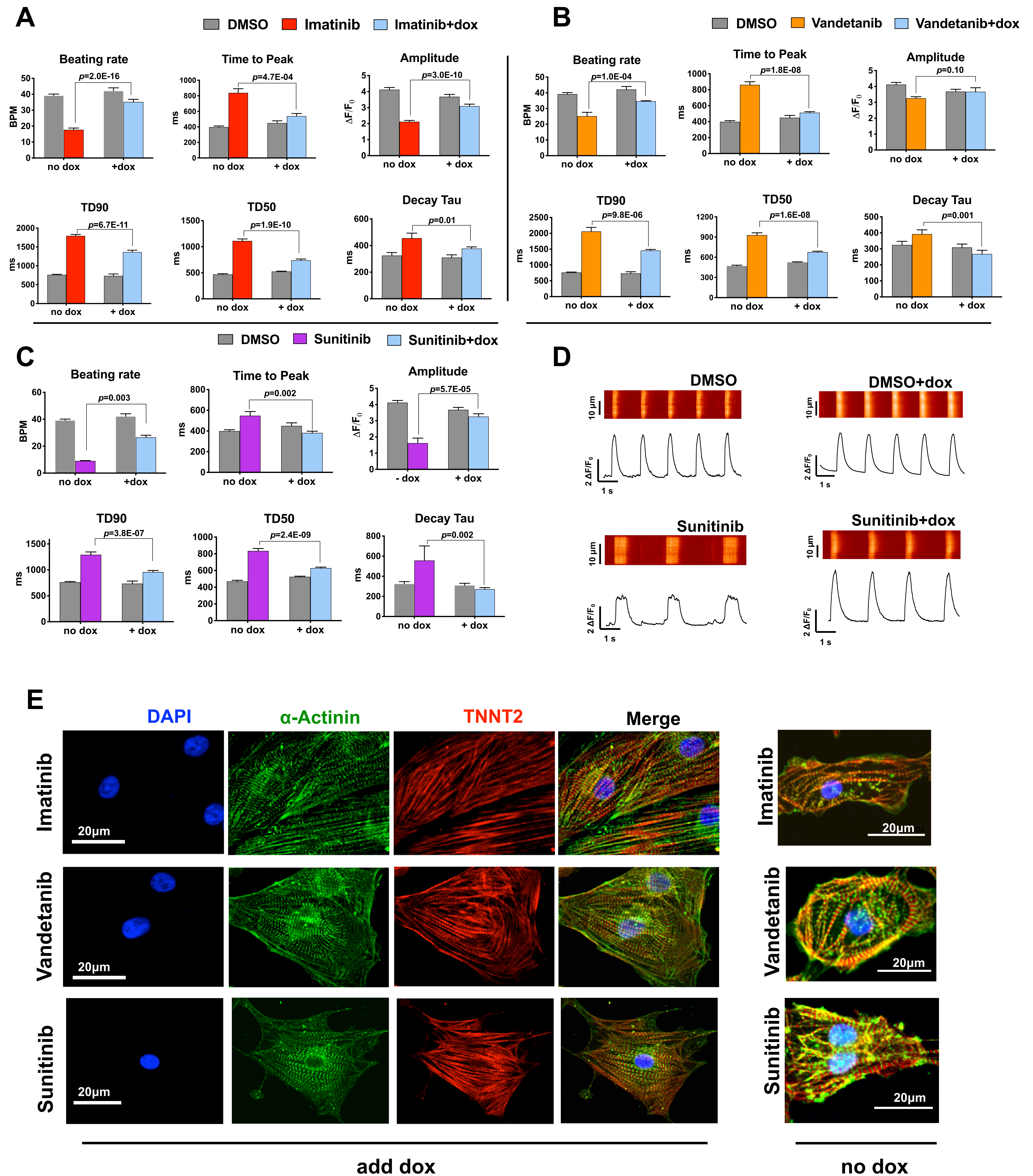


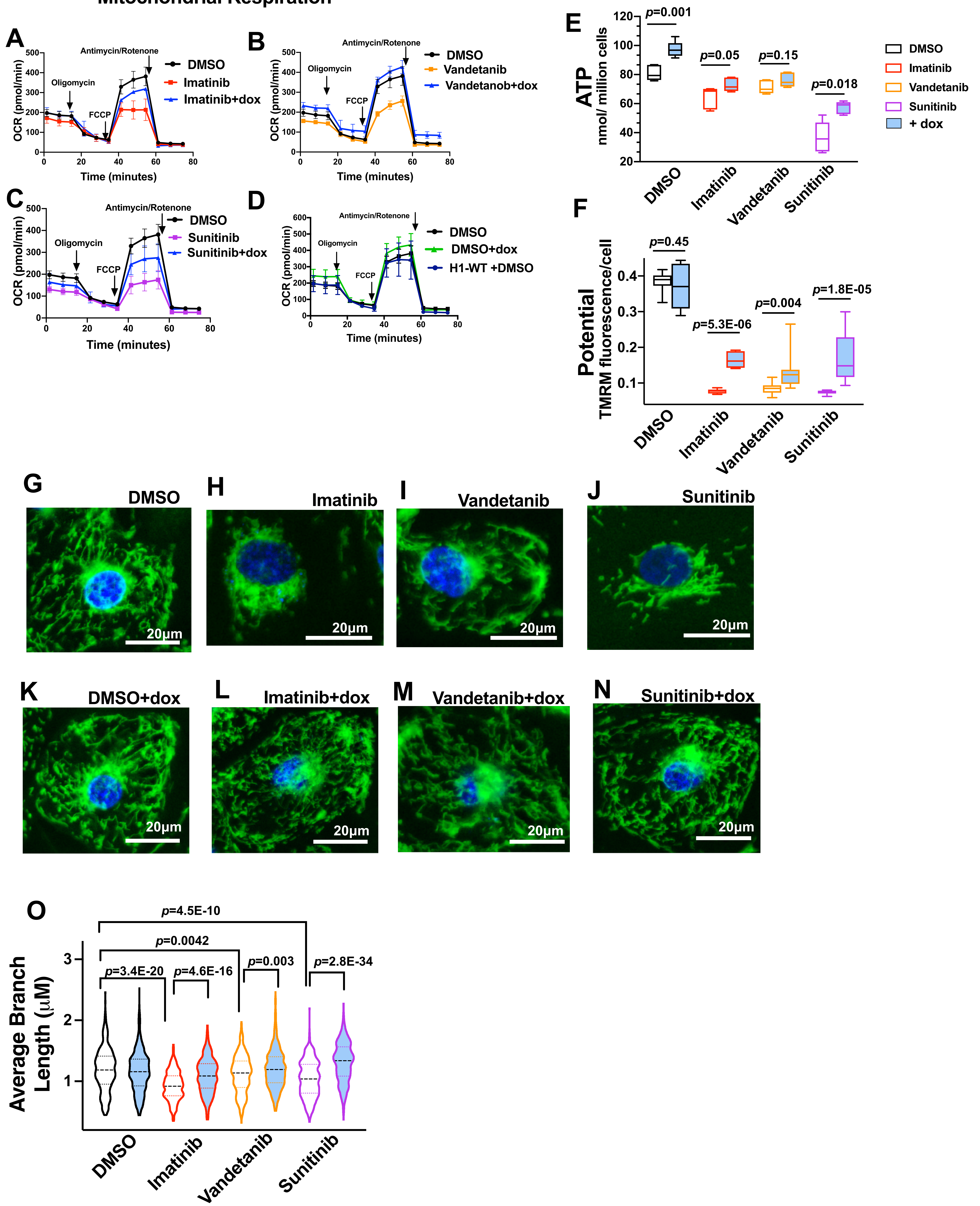
Figure 6**Mitochondrial Respiration**

Figure 7

