



AMPK β 1 and AMPK β 2 define an isoform-specific gene signature in human pluripotent stem cells, differentially mediating cardiac lineage specification

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AMP-activated protein kinase (AMPK) is a key regulator of energy metabolism that phosphorylates a wide range of proteins to maintain cellular homeostasis. AMPK consists of three subunits: α , β , and γ . AMPK α and β are encoded by two genes, the γ subunit by three genes, all of which are expressed in a tissue-specific manner. It is not fully understood, whether individual isoforms have different functions. Using RNA-Seq technology, we provide evidence that the loss of AMPK β 1 and AMPK β 2 lead to different gene expression profiles in human induced pluripotent stem cells (hiPSCs), indicating isoform-specific function. The knockout of AMPK β 2 was associated with a higher number of differentially regulated genes than the deletion of AMPK β 1, suggesting that AMPK β 2 has a more comprehensive impact on the transcriptome. Bioinformatics analysis identified cell differentiation as one biological function being specifically associated with AMPK β 2. Correspondingly, the two isoforms differentially affected lineage decision toward a cardiac cell fate. Although the lack of PRKAB1 impacted differentiation into cardiomyocytes only at late stages of cardiac maturation, the availability of PRKAB2 was indispensable for mesoderm specification as shown by gene expression analysis and histochemical staining for cardiac lineage markers such as cTnT, GATA4, and NKX2.5. Ultimately, the lack of AMPK β 1 impairs, whereas deficiency of AMPK β 2 abrogates differentiation into cardiomyocytes. Finally, we demonstrate that AMPK affects cellular physiology by engaging in the regulation of hiPSC transcription in an isoform-specific manner, providing the basis for further investigations elucidating the role of dedicated AMPK subunits in the modulation of gene expression.

The serine/threonine kinase 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a central player in cellular energy homeostasis and stress response (1, 2). Upon a

reduction of intracellular energy levels that is reflected by an increased AMP/ATP ratio, AMPK becomes activated and induces the phosphorylation of substrate proteins. Consequently, catabolic pathways are promoted while anabolism is inhibited to restore energy balance. Because of its fundamental role in various metabolic processes, AMPK is considered a promising target for the treatment of several pathologies, most prominently metabolic disorders, but also cancer and inflammatory diseases (3, 4).

AMPK is a heterotrimeric protein complex that is composed of a catalytic α subunit and two regulatory subunits β and γ . The α subunits contain the kinase domain and a phosphorylation site that is required for activation of the protein. AMPK activity is supported by myristoylation occurring at the β subunits (5), which additionally comprise a carbohydrate-binding module. The γ subunits are responsible for nucleotide binding and thereby allow AMPK to adapt to changes in the energetic requirement of the cell. Although two genes each encode the α and β subunits, three γ isoforms exist. Consequently, 12 different heterotrimeric combinations can be formed, which are expressed in a tissue- and species-specific manner. Whereas α 1, β 1, and γ 1 seem to be ubiquitously expressed, availability of the other isoforms is more differential (6), whereby different AMPK trimers vary in their physiological properties. Along this line, AMPK protein complexes containing the β 2 instead of the β 1 isoform possess a greater binding affinity to glycogen (7) and an increased activity following sumoylation (8). Although the specificity of individual AMPK isoforms and distinct trimer combinations is gaining more and more attention, the isoform-specific functions of AMPK are not completely understood.

Besides phosphorylating transcription factors and transcriptional coactivators, including peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), FOXO proteins, and others (9–12), AMPK can impact gene expression by indirect mechanisms (13, 14). Additionally, AMPK was found to directly associate with chromatin and to trigger histone modifications (15–17). This, however, requires nuclear localization of the protein, which also depends on the trimer composition (18–20) and can be achieved by posttranslational modifications (21, 22).

In the heart, AMPK plays an important role in the adaption of cellular energy balance by modulating glucose and fatty acid metabolism. It has been extensively studied in the context of

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cardiac dysfunction, particularly in ischemia-reperfusion injury and hypertrophy (23, 24). Although a high prevalence of α 2- and β 2-containing AMPK trimers is described for human heart tissue, the only subunit that could clearly be associated with human pathologies is γ 2 (6). The metabolic characteristics of selective AMPK complexes and single isoforms in different organs are gaining more and more attention, especially with regard to the development of pharmacological modulators of AMPK activity. In contrast to pan-AMPK activators or inhibitors, isoform-selective compounds take advantage of the specificity of AMPK complexes. They feature high kinase selectivity and reduced off-target effects. However, the physiological consequences resulting from the tissue-selective composition of AMPK complexes are not fully understood so far.

In the present study, we investigated the functional differences between β 1- and β 2-containing AMPK trimers in human induced pluripotent stem cells (hiPSCs), providing evidence that the lack of either subunit differently alters the gene expression profile. As a consequence, the differentiation toward the cardiac lineage is affected in an isoform-specific manner, indicating different physiological functions of AMPK β 1 and β 2 isoforms.

Results

Human induced pluripotent stem cells as a model to investigate isoform-specific functions of AMPK β 1 and AMPK β 2

To investigate cellular effects that are differentially mediated by AMPK β 1 and AMPK β 2, we used hiPSCs harboring a gene knockout for one or the other isoform. The WT cell line was selected based on its approximately equal expression of the two AMPK β subunits (Fig. 1A) and KO cell lines were created by CRISPR/Cas9-mediated gene modification (Fig. 1B). *PRKAB1*^{-/-} cells were generated by insertion of one base pair within exon 1, resulting in an in-frame stop codon. For the deletion of *PRKAB2*, a fragment of exon 2 was excised, generating a stop codon in exon 2. The growth behavior as well as the morphology of the cell colonies did not differ between *PRKAB1*^{-/-} or *PRKAB2*^{-/-} hiPSCs and WT cells (Fig. 1C). Importantly, deficiency of neither AMPK β 1 nor AMPK β 2 resulted in a compensatory up-regulation of the respective other isoform (Fig. 1D). Of note, protein levels of AMPK α 1 and AMPK α 2 were unchanged (Fig. 1E). We further confirmed that basal activity of AMPK was not affected by the knockout of either AMPK β 1 or AMPK β 2. All cell lines tested showed levels of phosphorylated AMPK and of acetyl-CoA carboxylase (ACC) similar to those in WT hiPSCs (Fig. 1, F and G).

AMPK β 1- and AMPK β 2-deficient undifferentiated hiPSCs show isoform-specific gene expression

The transcriptomes of WT hiPSCs as well as two clones of each KO cell line were analyzed by RNA-Seq. For that, as for the experiments described before, two knockout cell lines for each genotype were used. Cell lines were generated from single clones after gene knockout of either *PRKAB1* or *PRKAB2* to ensure the cultivation of individual cell populations. RNA-Seq

was furthermore performed in quadruplicates to allow for solid statistical evaluation of the data. Although 404 genes were regulated with a \pm -fold change >1.3 (p -value <0.05) relative to WT in *PRKAB2*^{-/-} hiPSCs, this applied to only 132 genes in *PRKAB1*^{-/-} hiPSCs (Fig. 2A). To identify the cellular effects that might arise from these differences, major downstream pathways of AMPK were investigated. However, analysis of the phosphorylation status of regulatory associated protein of mTOR (Raptor) (Fig. S1A) and tuberous sclerosis complex 2 (TSC2) (Fig. S1B) revealed no significant changes. Genes being associated with glucose and lipid metabolism did not vary between *PRKAB1*^{-/-} or *PRKAB2*^{-/-} and WT hiPSCs (data not shown). Oxidative metabolism also appeared to be unchanged under basal conditions, as expression levels of *PPARGCIA* and, consistently, visualization of mitochondria by MitoTracker staining (Fig. S1C) did not reveal any differences between the genotypes. In line with this, β -oxidation of fatty acids in the absence of glucose did not differ between WT and AMPK β 1- or AMPK β 2-deficient cells (Fig. S1D). Corresponding to our previous data, comparative pathway analysis of the gene sets did not show major differences between WT and any of the KO hiPSC lines (data not shown). Although, because of the low number of regulated genes, no solid GO enrichment analysis could be performed in *PRKAB1*^{-/-} cells, we could identify several biological processes that might be affected by the lack of AMPK β 2. Thereby, a large data set of the regulated genes in *PRKAB2*^{-/-} hiPSCs could be assigned to cell differentiation (Fig. 2B). As we did not detect differences between the two clones of identical genotypes, further analyses were carried out with one clone of each genotype. Gene expression of the pluripotency markers POU Class 5 Homeobox 1 (*POU5F1/OCT4*), SRY-box transcription factor 2 (*SOX2*), and *NANOG* was analyzed in undifferentiated hiPSCs and was not altered between the three cell lines (Fig. 2C). However, the independent differentiation into the three germ layers revealed lower expression of the mesoderm and endoderm markers T-box transcription factor T (*T/Brachyury*) and *SOX17* in *PRKAB2*^{-/-} relative to WT cells (Fig. 2D). In contrast, mRNA levels of the ectoderm marker *PAX6*, although lower in undifferentiated cells, was significantly higher in cells derived from *PRKAB2*^{-/-} than in those from WT hiPSCs. *PRKAB1*^{-/-} hiPSCs consistently showed a differentiation potential similar to WT cells. Together, our data indicate that AMPK β 1- and AMPK β 2-containing AMPK trimers attain unique physiological effects that, in hiPSCs, affect differentiation potential. Specifically, the availability of AMPK β 2 seems to be required for the development toward the mesodermal and endodermal lineage.

Deficiency of *PRKAB1* impairs, whereas loss of *PRKAB2* abrogates, differentiation into cardiomyocytes (CMs)

To further evaluate the impact of either AMPK β 1 or AMPK β 2 deficiency on mesoderm differentiation, we applied all three cell lines to a standardized cardiomyocyte differentiation protocol (Fig. 3A). Protein expression of AMPK β 1 and of AMPK β 2 increased during differentiation in WT as well as in both knockout cell lines, whereas no continuous differences in

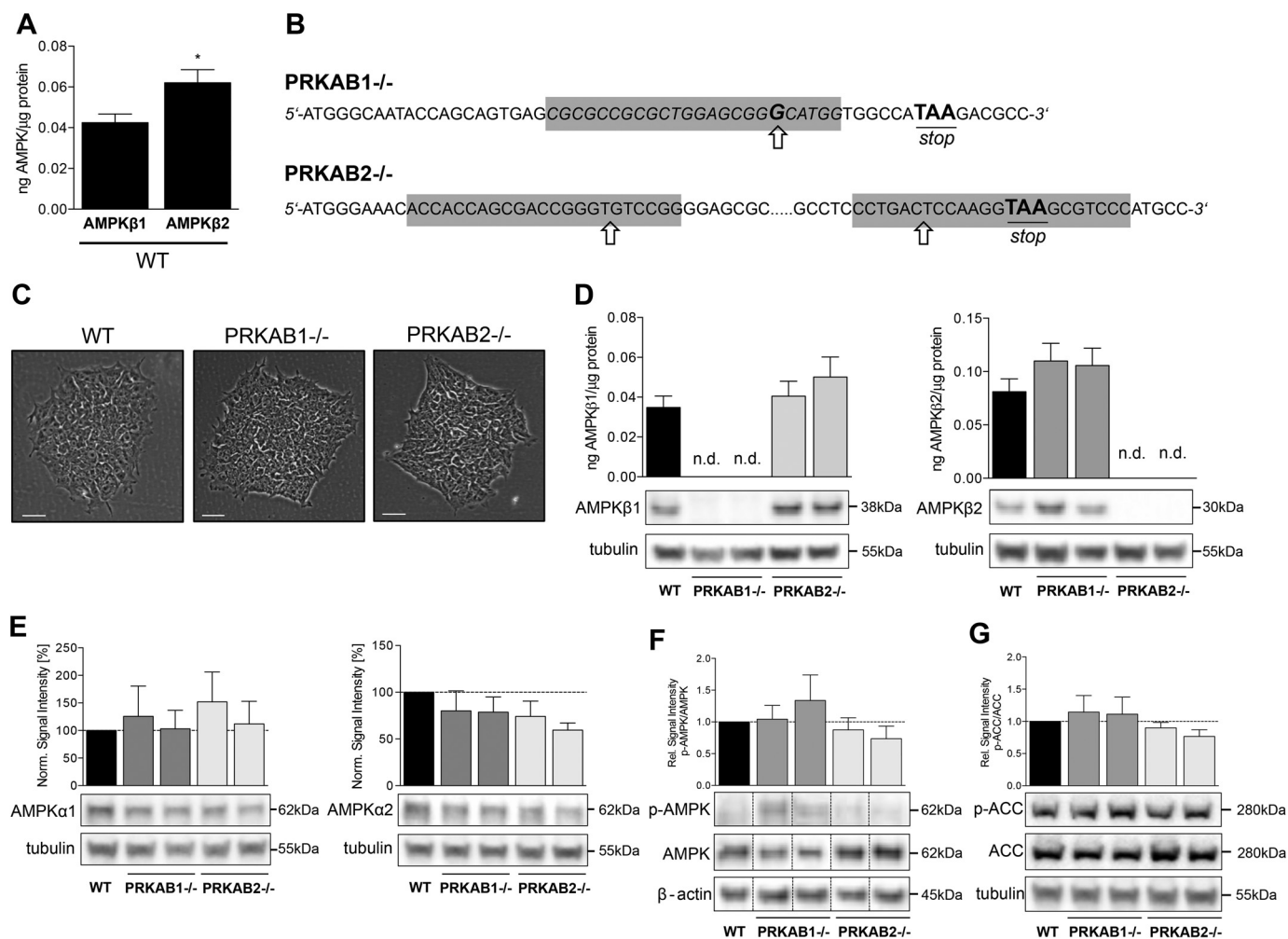


Figure 1. Characterization of the cellular model used to study isoform-specific effects of AMPK. A, amount of AMPK β 1/ β 2 protein in WT hiPSCs determined by Western blotting and calculated by means of a standard curve of recombinant AMPK ($n = 10$ independent experiments, *, $p < 0.05$). B, KO strategy used to generate PRKAB1 $^{-/-}$ and PRKAB2 $^{-/-}$ hiPSCs. Gray boxes frame gRNA sequences, arrows indicate cutting sites. One base insertion is in bold. C, representative phase-contrast images of cell colonies (scale bars, 50 μ m). D, Western blots and quantification of total AMPK β 1 in WT and PRKAB2 $^{-/-}$ hiPSCs (left) and AMPK β 2 in WT and PRKAB1 $^{-/-}$ hiPSCs (right). Two KO clones per genotype were analyzed ($n = 8$ independent experiments; n.d., not detectable). E, Western blots and quantification of AMPK α 1 (left) and AMPK α 2 (right) in WT, PRKAB1 $^{-/-}$, and PRKAB2 $^{-/-}$ hiPSCs. Signal intensity is shown relative to WT ($n = 4/6$ independent experiments, two clones per KO cell line were analyzed and plotted as individual bars). F and G, representative Western blotting and quantification for phosphorylated AMPK in WT, PRKAB1 $^{-/-}$, and PRKAB2 $^{-/-}$ hiPSCs. Signal intensity is shown relative to WT ($n = 6/5$ independent experiments). Dotted lines in (F) indicate blot splicing, to delete irrelevant sample lanes.

protein levels were observed between WT and PRKAB1 $^{-/-}$ or PRKAB2 $^{-/-}$ hiPSCs (Fig. S2A). Thereby, the appearance of lineage-specific marker genes was monitored (Fig. 3B). SOX2 and OCT4 as well as early differentiation markers including the mesendoderm-associated gene *T* and the homeobox protein *MIXL* became massively down-regulated over time in WT and PRKAB1 $^{-/-}$ hiPSCs. Strikingly, in PRKAB2 $^{-/-}$ cells, expression of these marker genes remained rather high, whereas genes that indicate early cardiac cell fate such as islet-1 (*ISL1*) and myocyte enhancer factor 2C (*MEF2C*) showed much lower induction than in WT. The same was true for *TNNT2*, encoding for cardiac troponin T2 (cTnT) as well as for signal regulatory protein alpha (*SIRPA*), two markers commonly used to identify cardiac cell populations. Unlike PRKAB2 $^{-/-}$ cells, WT and PRKAB1 $^{-/-}$ hiPSCs strongly increased *TNNT2* transcription starting from around day 6 of differentiation. The differences in gene expression were equally translated to con-

siderably lower protein content (Fig. 3, C–E and Fig. S2B). To evaluate differentiation efficiency, we quantified the number of cells co-expressing cTnT and α -actinin, one feature of mature and functional CMs. On average 30, 18, and 1.4% cTnT- and α -actinin-positive cells were obtained after metabolic selection and 13 days of maturation in WT, PRKAB1 $^{-/-}$, and PRKAB2 $^{-/-}$ cells, respectively (Fig. 3E). Consistently, expression of genes encoding for sarcomere and calcium-handling proteins, a prerequisite for cardiac contractility, was lower in PRKAB1 $^{-/-}$ cells relative to WT and only minimal in PRKAB2 $^{-/-}$ cells (Fig. S3A). Interestingly, phosphorylation of ACC was similar in all three cell lines throughout differentiation (Fig. S2C), suggesting that AMPK activation did not significantly change. In search for regulatory mediators of cardiac development that might be affected by the deficiency of AMPK β 1 and β 2, we found that the up-regulation of the key cardiac transcription factors including GATA4 and

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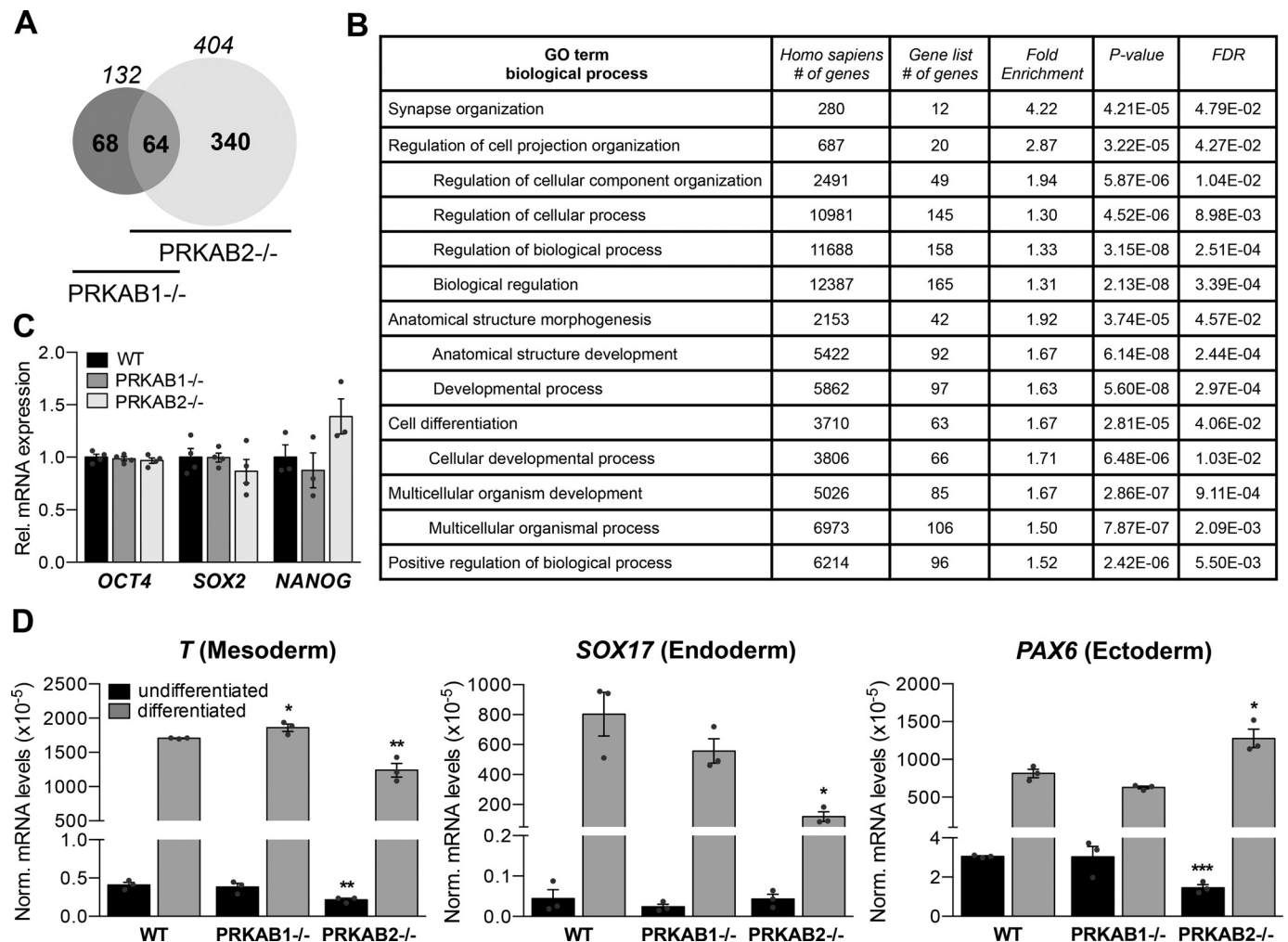


Figure 2. Loss of AMPK β 1 and AMPK β 2 alters the transcriptome of undifferentiated hiPSCs. A, Venn diagram of RNA-Seq data illustrating genes being differentially expressed in PRKAB1 $^{-/-}$ and PRKAB2 $^{-/-}$ relative to WT hiPSCs (≥ 2 -fold change for two clones of each genotype). Experiment was done in four replicates. B, GO term analysis of genes being down-regulated by FC > 1.3 in PRKAB2 $^{-/-}$ hiPSCs relative to WT. Annotation dataset was GO biological process (FDR $p < 0.05$). C, qRT-PCR for *OCT4*, *SOX2*, and *NANOG* in WT, PRKAB1 $^{-/-}$, and PRKAB2 $^{-/-}$ hiPSCs. Data are normalized to WT levels ($n = 3/4$ independent experiments). D, qRT-PCR analysis of mesoderm (*T*), endoderm (*SOX17*), and ectoderm (*PAX6*) markers in undifferentiated hiPSCs and after lineage-specific differentiation ($n = 3$ independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

NKX2.5 were compromised, especially in PRKAB2 $^{-/-}$ hiPSCs (Fig. 3F and Fig. S3B).

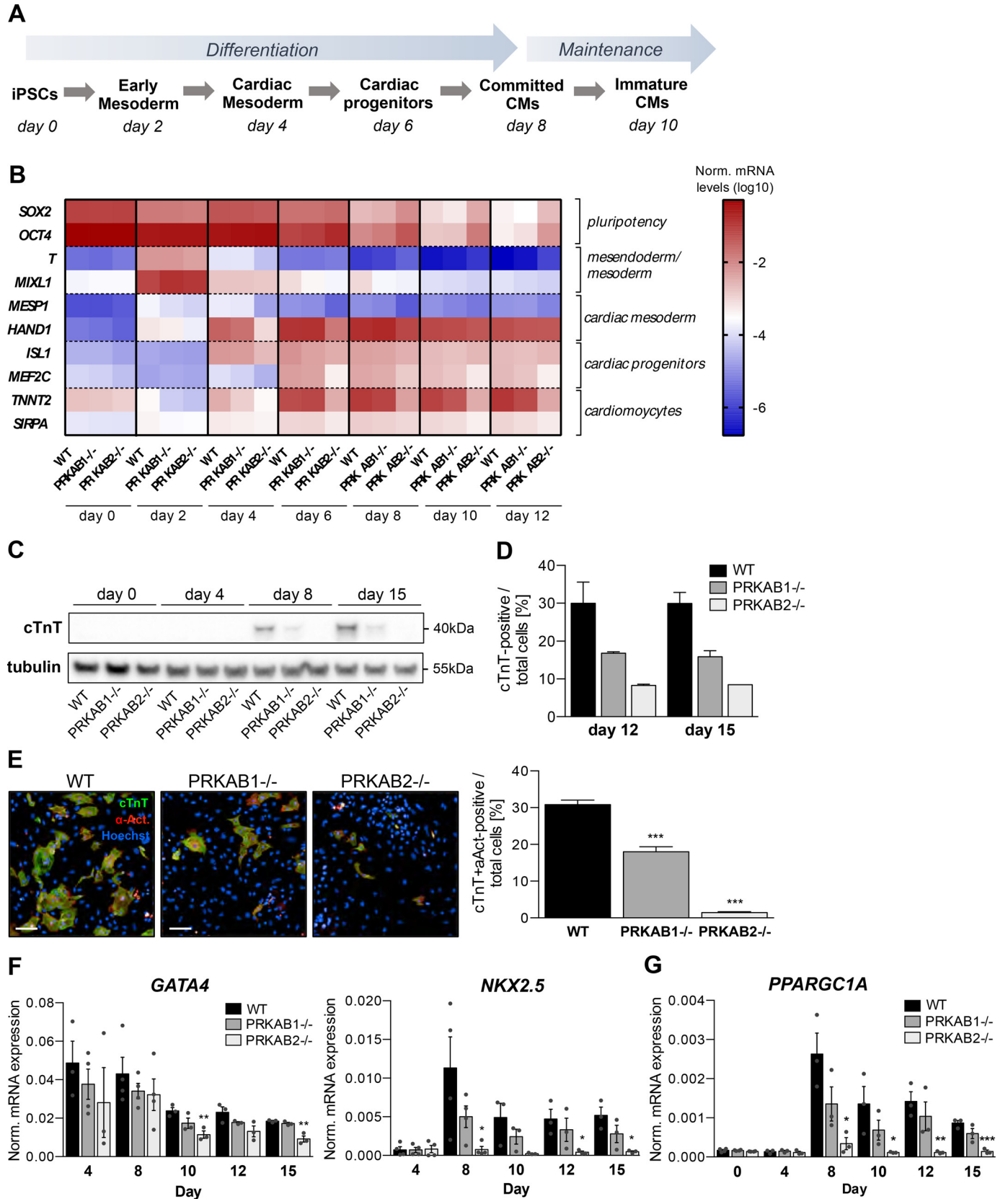
The AMPK target PGC-1 α , encoded by *PPARGC1A*, is essential for proper development of CMs from embryonic stem cells (25) and was shown to be part of a co-expression network defining cardiac identity (53). *PPARGC1A* expression was significantly lower in PRKAB2 $^{-/-}$ cells starting from day 8 onward (Fig. 3G). Furthermore, a number of miRNAs being required for the activation of CM-specific genes and thus for fine-tuning lineage specification were analyzed (26–28). Their expression pattern mirrored that of the cardiomyocyte-specific genes (Fig. S4). Taken together, these data indicate that AMPK β 1 and AMPK β 2 differentially affect the differentiation potential of hiPSCs toward the cardiac lineage, involving various regulatory mediators. The lack of AMPK β 1 affects cardiac differentiation at the progenitor stage, impairing maturation into fully functional cardiomyocytes. In contrast, PRKAB2-deficient hiPSCs hardly give rise to cTnT-positive cells, suggesting that AMPK β 2-containing isoforms are required for early lineage specification.

Lack of AMPK β 2 impairs mesendoderm lineage decision of hiPSCs

The loss of pluripotency is an essential prerequisite for the differentiation of stem cells into any lineage. Following up on our previous experiments that suggest deficiencies in the development of PRKAB2 $^{-/-}$ hiPSCs already at early time points, we observed a higher number of Oct-3/4-positive cells at day 8 of differentiation in PRKAB2 $^{-/-}$ relative to WT and PRKAB1 $^{-/-}$ cells (Fig. 4, A and B). Similar results were obtained by qRT-PCR for *OCT4* (Fig. 4C) and *SOX2* (Fig. 4D). Based on the fact that AMPK β 2-deficient hiPSCs seem to be specifically limited in their differentiation potential toward mesoderm and endoderm (Fig. 2H), we speculated that significant impairments might manifest already before the time point when both lineages separate from a common precursor. Consistently, the mesoderm/mesendoderm marker Brachyury/*T* showed markedly lower expression at early stages of differentiation, whereas levels appeared to be slightly higher at later time points compared with WT as well as PRKAB1 $^{-/-}$ hiPSCs (Fig. 4E). Expression of *MESP1*, however, a marker for cardiac

mesoderm, was barely detectable and below levels of WT and PRKAB1 $^{-/-}$ cells throughout differentiation (Fig. 4F). The process of stem cell development toward mesendoderm is

mediated by a coordinated crosstalk of different signaling pathways including transforming growth factor- β , Wnt, and fibroblast growth factor signaling (29–32). Consequently, the



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expression of some key mediators responsible for the coordinated translation of external signals into a transcriptional response (33–35) was analyzed in *PRKAB2*^{-/-} and WT hiPSCs at day 2 of differentiation, the time point at which the cells pass the mesendoderm stage (Fig. 3B). Expression of *SMAD2*, *SMAD4*, and *FOXH1*, which modulate BMP3 and activin signaling, as well as of *CTNNB1* encoding for the Wnt mediator β -catenin, were repressed in *PRKAB2*^{-/-} relative to WT hiPSCs (Fig. 4G). Altogether, these data predict that lack of AMPK β 2 impairs already early stages of mesendoderm differentiation.

AMPK β 1 and AMPK β 2 are required for the expression of pluripotency and cardiac lineage genes

To confirm that the alterations in cardiac gene expression can be attributed to the lack of either AMPK β 1 or AMPK β 2, we re-expressed the missing isoform in the respective KO cells. For that, *PRKAB1* and *PRKAB2* cDNA was cloned into a lentiviral vector under the control of the EF-1 α promoter (Fig. 5A). Successful integration and expression of the protein was confirmed (Fig. 5B). Although the rescue of AMPK β 1 and AMPK β 2 protein expression was only around 10 and 5% of the WT levels, the partial reconstitution of AMPK β 2 was sufficient to significantly decrease *OCT4* as well as *SOX2* expression relative to *PRKAB2*^{-/-} cells starting from day 4 of differentiation (Fig. 5C). This suggests that even a low amount of AMPK β 2 is sufficient to initiate cell differentiation. Consistently, expression levels of *GATA4* and *NKX2.5* were increased in cells re-expressing AMPK β 1 and AMPK β 2 (Fig. 5D). Together, these experiments illustrate the isoform-specific function of AMPK on the expression of dedicated genes, thereby modulating cell physiology in different ways.

Discussion

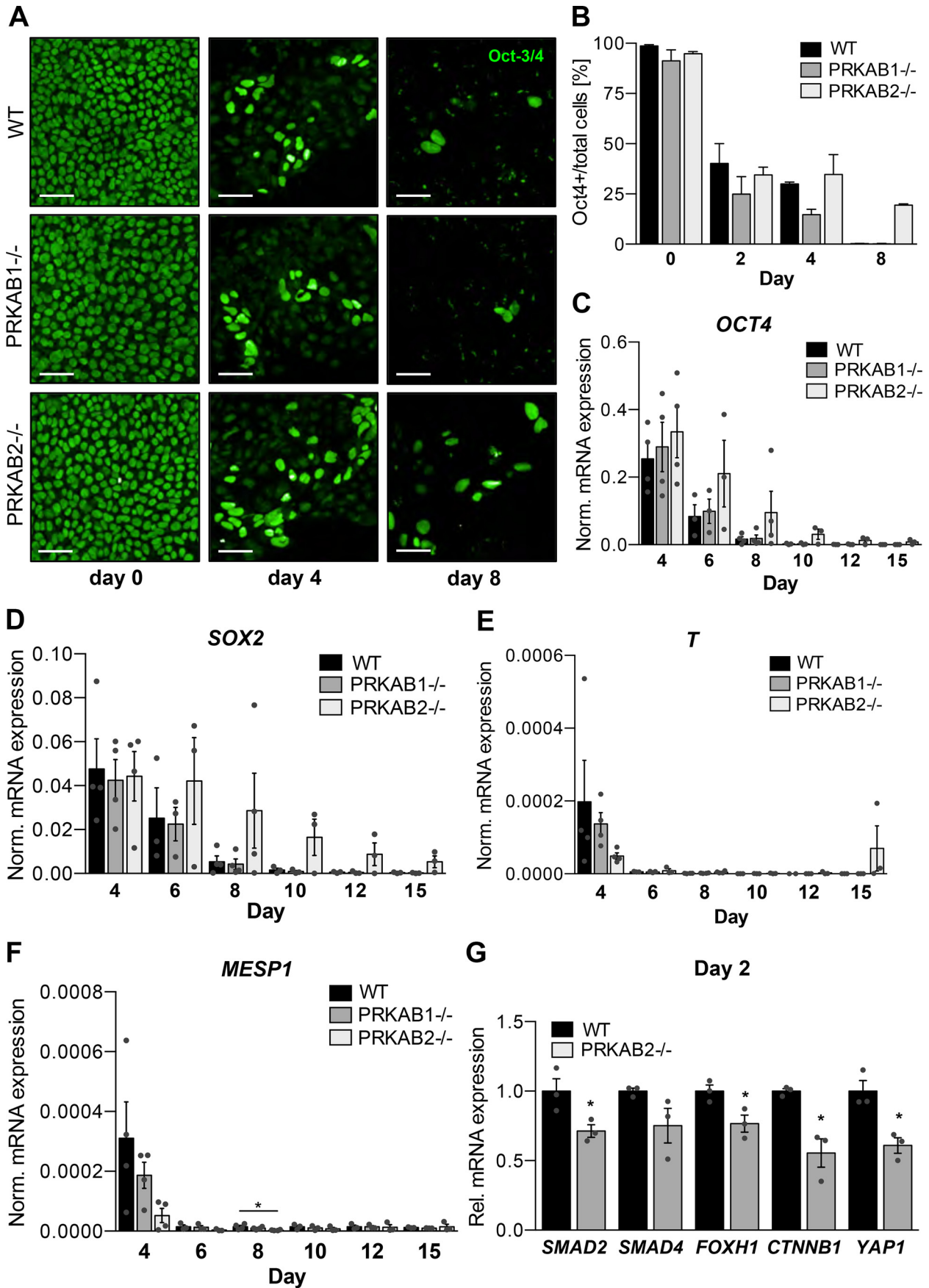
In the present study, we demonstrate that AMPK heterotrimers containing either the β 1 or the β 2 subunit in the protein complex account for distinct gene expression profiles in human iPSCs and, as a consequence, differentially affect downstream physiological processes. Specifically, we show that AMPK β 1 and β 2 influence cardiac differentiation potential in an isoform-specific manner. Lack of *PRKAB1* impairs, but does not prevent, the development of CMs. In contrast, *PRKAB2* deficiency nearly abrogates cardiac differentiation most likely by prohibiting lineage decision at the mesendoderm stage.

The hiPSC line used in our study was chosen because it possesses similar amounts of both AMPK β isoforms. Although we failed to generate iPSCs lacking both subunits, single knockout of either AMPK β 1 or AMPK β 2 was successful. The knockout of neither *PRKAB1* nor *PRKAB2* caused a significant counter-

regulation of other AMPK subunits, excluding compensatory mechanisms masking AMPK β -specific effects. RNA-Seq analysis of WT, *PRKAB1*^{-/-}, and *PRKAB2*^{-/-} hiPSCs revealed major differences in their transcriptomes and thereby for the first time provides evidence for an AMPK β isoform-specific gene expression profile. The WT cells used in our study were not transfected with a control gRNA. However, as differences in gene expression were observed in two clones of each genotype relative to WT and also between cells lacking one or the other AMPK β isoform, off-target effects caused by the knockout procedure could be excluded. Furthermore, reconstitution of the missing AMPK subunit restored gene expression in the respective knockout cell line, additionally supporting our conclusion that the differences between WT and KO hiPSCs are because of isoform-specific effects on gene expression. Basal phosphorylation of AMPK was the same in WT as in both KO cell lines. With that, the changes in gene expression were most likely not because of variations in overall cellular AMPK activity, but rather caused by the downstream effects that selectively depend on the presence or selective activation of AMPK β 1 and β 2. The two isoforms possess only 71% sequence homology (36), so that β 1- and β 2-containing AMPK trimers undergo different posttranslational modifications and vary in their affinity to carbohydrates. This might result in different substrate affinities and subcellular localization of the protein (7, 8, 18), which could provide the molecular basis for the functional differences between AMPK isoforms containing either the β 1 or the β 2 subunit observed in our study. Another possibility is that β 1- and β 2-containing trimers might be differentially regulated. Evidence for an isoform-selective activation of AMPK has been obtained in skeletal muscle (37). AMPK can directly affect gene expression via several mechanisms and by the interaction with different factors. For example, it could be shown that AMPK is able to phosphorylate a number of transcription factors and transcriptional coactivators including PGC-1 α , FOXO3, CREB proteins and others, thereby impacting gene expression profiles in different tissues (9–11). Furthermore, AMPK was proven to have functions in histone modification and epigenetic mechanisms as histone H2B and p300 were identified as substrates of AMPK (15, 16). At this point, we can only speculate that the selection of AMPK β 1 or AMPK β 2 in the protein complex intervenes with those diverse and multifactorial mechanisms and, especially in stem cells, directs cell fate decision. However, further studies will be needed to decipher the role of dedicated AMPK subunits on stem cell physiology and function.

Analysis of the differentially expressed genes identified by RNA-Seq did not allow an assignment of any pathway to either β 1- or β 2-containing trimers, suggesting that these discrepancies do not significantly impact downstream effects of AMPK in the pluripotent state. This corresponds to the suggestion of

Figure 3. AMPK β 2-deficient hiPSCs are unable to develop into contracting CMs. A, developmental timeline of hiPSCs during cardiac differentiation. B, qRT-PCR analysis illustrated as heat map of lineage-specific genes in WT, *PRKAB1*^{-/-}, and *PRKAB2*^{-/-} hiPSCs. Normalized expression levels are calculated as log₁₀ ($n = 3/5$ independent experiments). C, Western blots for cTnT at day 0, 4, 8, and 15 of differentiation. D, quantification of IF staining for cTnT at day 12 and day 15. Differentiation was performed in duplicates; ≥ 800 cells were analyzed per experiment and condition. E, representative images of IF staining for cTnT and α -actinin, quantification of cTnT- and α -actinin-positive cells at day 28. Quantification is calculated as mean of two independent differentiations with 7/4 replicates. ≥ 600 cells were analyzed per experiment and condition (scale bars are 100 μ m; ***, $p < 0.0001$). F, qRT-PCR analysis of *GATA4* and *NKX2.5* in WT, *PRKAB1*^{-/-}, and *PRKAB2*^{-/-} hiPSCs ($n = 3/4$ independent experiments). G, time course of mRNA levels of *PPARGC1A* in WT, AMPK β 1- and AMPK β 2-deficient cells ($n = 3$ independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



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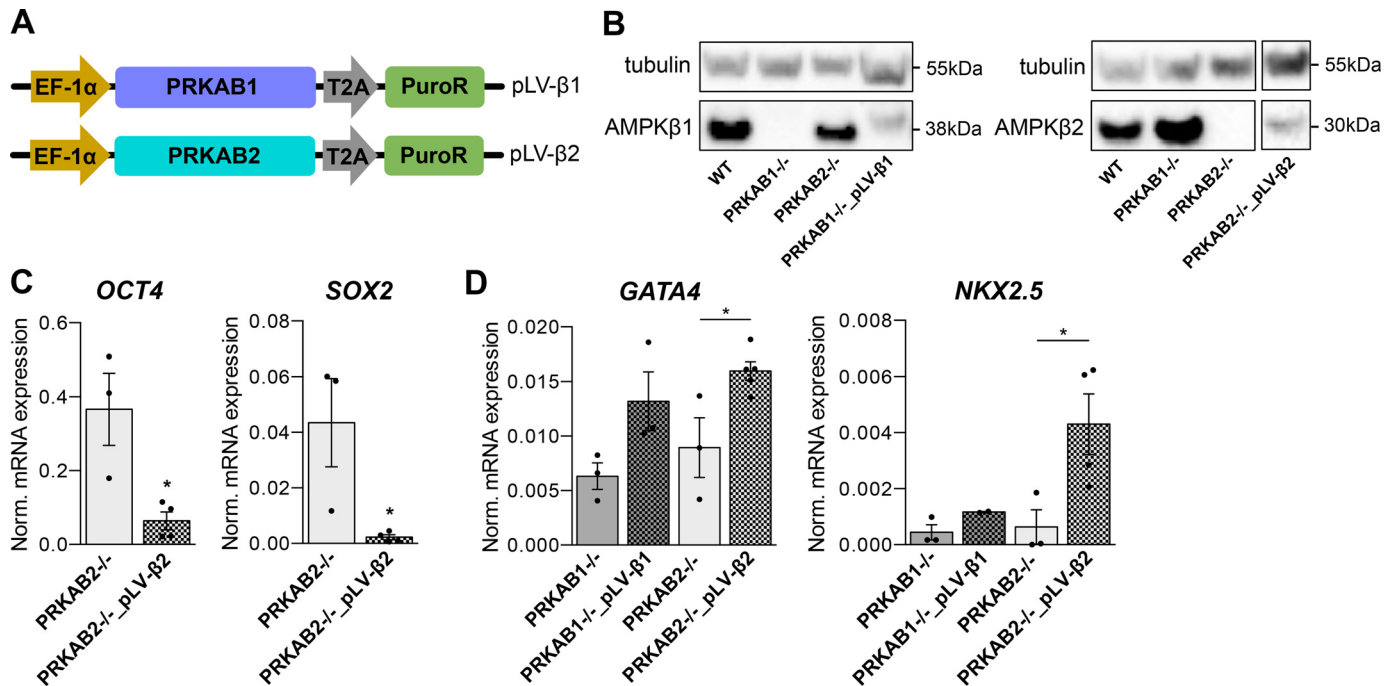


Figure 5. Re-expression of AMPK β 1 and AMPK β 2 partially rescues expression of cardiac lineage-specific genes. A, schematics of the constructs used for reconstitution of AMPK β 1 and AMPK β 2 in the respective KO hiPSC line. B, Western blots for AMPK β 1 (left) and AMPK β 2 (right) of WT, PRKAB1^{-/-}, and PRKAB2^{-/-} hiPSCs and of infected hiPSCs. C, qRT-PCR of OCT4 and SOX2 in PRKAB2^{-/-} and pLV- β 2-infected PRKAB2^{-/-} hiPSCs at day 4 of differentiation ($n = 3/4$ independent experiments). D, qRT-PCR analysis of KO hiPSCs and hiPSCs re-expressing PRKAB1 or PRKAB2 for GATA4 at day 2 ($n = 3/5$ independent experiments) and NKX2.5 at day 4 (PRKAB1^{-/-} and PRKAB1^{-/-}_pLV- β 1 are $n = 3/2$, PRKAB2^{-/-} and PRKAB2^{-/-}_pLV- β 2 are $n = 3/4$ independent experiments; *, $p < 0.05$).

Young *et al.* (38) that AMPK plays a minor role in undifferentiated stem cells under basal conditions. However, several developmental processes and cell differentiation GO terms could specifically be associated with AMPK β 2, indicating that the consequences induced by differential gene expression predominantly emerge under differentiating conditions. Trilineage differentiation experiments confirmed this finding. Although cell fate decision into mesoderm and endoderm was reduced in AMPK β 2-deficient hiPSCs relative to WT, ectoderm development was facilitated. These findings substantiate previous results, indicating that AMPK activity is not required for the lineage decision toward ectoderm (38).

However, AMPK has been shown to be involved in the differentiation of stem and progenitor cells (39–41) but, unlike for the two isoforms of AMPK α , any specificity for either β 1- or β 2-containing trimers in respect to cell fate determination was not described before. Our three stem cell genotypes varied in their cardiac differentiation potential, which was mirrored by major discrepancies in the expression of cardiac lineage markers as well as genes being associated with sarcomere function. Expression levels of AMPK β 1 as well as of AMPK β 2 continuously increased between day 0 and day 15 of differentiation. Significant differences between WT and either of the knockout hiPSCs could only be detected at one time point of differentia-

tion in PRKAB2^{-/-} cells, suggesting that the lack of one β isoform does not induce a general mechanism leading to a compensatory up-regulation of the respective other β isoform. Literature data illustrating the expression pattern of AMPK β during cardiac development *in vivo* are rare. Kim *et al.* (42) investigated this aspect in mice and observed a drop of the expression of both AMPK β 1 and β 2 from E15 to day 1 after birth as well as to adulthood. However, those studies are hardly comparable to human *in vitro* models as species-specific differences are likely. Furthermore, *in vitro* stem cell models poorly represent the *in vivo* cardiovascular environment (43–45). To our knowledge, our study for the first time investigates the dedicated impact of AMPK β isoforms on human cardiac development in a stem cell-based model. Isoform-specific functions of AMPK have been reported for cardiac physiology as AMPK activation modulates contraction of CMs by phosphorylating cTnT, leading to an increase in calcium sensitivity and contractility. This is mainly attributed to AMPK α 1 (46, 47). Moreover, AMPK is involved in the O-GlcNAcylation of certain proteins including troponin (48), which in turn has an impact on cTnT phosphorylation and cardiac function (49, 50), underlining the important role of AMPK in heart physiology. The switch from glycolysis to fatty acid oxidation as the main energy source is a prerequisite for proper differentiation and maturation of CMs

Figure 4. AMPK β 2 is important for lineage decision of hiPSCs. A, representative images of IF staining of Oct-3/4 in WT, PRKAB1^{-/-}, and PRKAB2^{-/-} cells at day 0, 4, and 8 of differentiation (scale bars are 50 μ m). B, quantification of IF staining showing number of Oct-3/4-positive cells/total cells. Bars are mean of 2/3 independent experiments; >1000 cells were analyzed per condition. C and D, time course of OCT4 (C) and SOX2 (D) expression analyzed by qRT-PCR ($n = 3/4$ independent experiments). E and F, gene expression of T (E) and MESP1 (F) in WT, PRKAB1^{-/-}, and PRKAB2^{-/-} cells at different time points during differentiation ($n = 3/4$ independent experiments). G, qRT-PCR analysis of genes being associated with mesoderm differentiation in WT and PRKAB2^{-/-} hiPSCs at day 2. Data shown are normalized to WT levels ($n = 3$ independent experiments).

and was shown to be indispensable for cell cycle exit and cardiac progenitor cell maturation of hPSC-derived cardiac organoids (51). The shift to oxidative phosphorylation requires mitochondrial network formation that is mediated by PGC-1 α , a well-described target of AMPK. PGC-1 α becomes up-regulated during cardiac development that is tightly associated and temporally dependent on the expression of the transcription factor NKX2.5 in hESC-derived cardiac progenitor cells (25). NKX2.5, together with other highly conserved transcription factors including GATA4, TBX5 and ZEB1, belongs to a regulatory network controlling heart development *in vivo* and cardiogenesis *in vitro* (52, 53). Lack of NKX2.5 in human ESC-derived CMs causes decreased expression of cardiac differentiation markers as well as impaired electrophysiology and contractile function (54). Overall, these reports correspond to our data. In WT cells, massive induction of NKX2.5 and TBX5 transcription and a simultaneous up-regulation of PPARC1A were observed at day 8 of differentiation that exactly coincides with an increase in the expression of TNNT2 and other sarcomere genes. Strikingly, lack of AMPK β 1 as well as AMPK β 2 showed major disturbances in this gene regulatory network, although to different extents. This is additionally confirmed by changes in the expression of specific miRNAs that are equally important for cardiac differentiation and physiology (26, 55–57), among others, altering the expression of cardiac transcription factors (58–60).

Although AMPK β 1-deficient hiPSCs maintain the ability to differentiate into CMs, lack of AMPK β 2 seems to abrogate it by attenuating the exit from pluripotency and the progression of differentiation. As the expression of effectors mediating activin, BMP and Wnt signaling, which coordinately promote the exit from pluripotency and subsequent mesoderm induction, was significantly decreased in cells lacking AMPK β 2, isoforms containing this subunit are obviously critical at very early stages of differentiation and required for cells to pass the mesendoderm stage. Wnt and activin signaling are modulated by YAP (61, 62), which can directly be phosphorylated by AMPK (63) and is under certain circumstances a target of O-GlcNAcylation (64). In addition, metabolite availability in hESCs can affect lineage decision via activation of AMPK (65).

Taken together, the mechanism underlying the phenotype observed in our stem cell model is most likely multifaceted and might include direct phosphorylation and posttranslational modifications of effector proteins at different levels of regulation. Besides an altered miRNA expression pattern, one can expect epigenetic modulations to interfere with an AMPK β -dependent regulatory network that finally defines the transcriptional program and metabolic adaptations of the cells. Moreover, subcellular localization of AMPK might be different (66). However, further investigations are needed to identify the mechanism(s) underlying the isoform-specific mRNA profiles in the present model.

In an apparent contradiction to our data, a major heart phenotype in whole body AMPK β 2 knockout animals has not been described (67, 68). Nevertheless, mice with a muscle-specific deletion of AMPK β 1/ β 2 showed cardiac dysfunction going along with decreased phosphorylation of troponin (69). We speculate that, *in vivo*, mechanisms compensating for the lack

of AMPK β 2 might exist that cannot equally take place during an *in vitro* differentiation protocol. Furthermore, a time-dependent regulation of AMPK activity that might take place during embryonic development is probably not equally adapted in a cell culture model. Along this line, it was shown recently that pharmacological activation of AMPK in cardiac progenitor cells under differentiation is neither sufficient to alter gene expression of differentiation and CM markers, nor promote PPARC1A transcription and mitochondrial biogenesis (70). A recent publication suggests an increase in AMPK activation during cardiac differentiation of hiPSCs, reaching highest levels of phosphorylation at days 9 and 11 followed by a drop of protein activation at day 14 (71). Our data do not confirm this finding, which might be because of differences in the differentiation protocol. Furthermore, analyzing the phosphorylation status of ACC to monitor AMPK activation during differentiation, we could not detect significant differences between WT and either of the KO cell lines. However, we cannot preclude that those differences might still exist but are not detectable because of insufficient sensitivity of Western blot analysis.

Although further investigations are required to precisely decipher the molecular mechanisms underlying the isoform-specific effects of AMPK β 1/ β 2 during differentiation, our data provide evidence that the AMPK isoforms exhibit different biological functions in hiPSCs. Whether those differences can also be observed in other cellular systems will be the substance of future studies.

Experimental procedures

Generation of hiPSC KO cell lines

PRKAB1 and PRKAB2 KO hiPSC lines were purchased as customized products from Applied StemCell. In brief, KO cell lines were generated from a WT hiPSC line (no. ASE-9202; ASC) by CRISPR/Cas9-mediated gene modification. For this, one (CGCGCCGCGCTGGAGCGGCANGG) or two (ACCA-CCAGCGACCGGGTGTCTCNGG, GGGACGCTTACCTTGG-AGTCNNG) gRNAs were utilized to create a frameshift KO mutation of the PRKAB1 or PRKAB2 gene, respectively. Individual iPSC colonies were expanded and KO was verified by sequence analysis. Positive staining for Oct-4, Sox-2, SSEA-4, TRA-1-60, and TRA-1-81 and for alkaline phosphatase as well as normal karyotype was approved by Applied StemCell.

Maintenance of human iPSC cells

Undifferentiated hiPSCs were cultured on Matrigel-coated plates seeded with mTeSRTMPlus medium; passaging of the cells was performed using ReLeSRTM (STEMCELL Technologies). Single cell suspensions were prepared using Accutase. After cell seeding, medium was substituted with ROCK inhibitor (BioVision) for 24 h.

Differentiation of hiPSCs and maintenance of CMs

Differentiation into mesoderm, ectoderm, and endoderm was performed using STEMdiffTM Trilineage Differentiation Kit (STEMCELL Technologies). For cardiac differentiation, the STEMdiffTM Cardiomyocyte Differentiation Kit (STEMCELL

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Technologies) was used following the manufacturer's protocol, starting with 1×10^5 cells/cm². For long-term maintenance of CMs, cells were selected in DMEM without glucose supplemented with 1% sodium lactate solution and 25 μ g/ml gentamicin (72) for 48 h. Afterward, cells were reseeded on Matrigel-coated cultureware using STEMdiffTM Cardiomyocyte Dissociation Kit (STEMCELL Technologies). Fat oxidation was measured as described (73).

Reconstitution of hiPSC KO cell lines

The coding sequences of PRKAB1, PRKAB2, and T2A-Puro were ordered as gBlocks from Integrated DNA Technologies. The respective PRKAB1/2-T2A-Puro in-frame fusion constructs were assembled via Golden Gate cloning technique. The sequence integrity was verified by Sanger sequencing. PRKAB1/2-T2A-Puro elements were excised with EcoRI/XbaI and cloned into a lentiviral vector under the EF1a promoter. VSV-G pseudotyped lentiviruses were produced with LV-MAX lentiviral production system according to standard protocols (Gibco).

RNA-Seq and bioinformatics analysis

Total RNA was purified using RNeasy kit (Qiagen) and sequencing was performed at ATLAS Biolabs GmbH. Bioinformatics analyses of RNA-Seq raw data were performed using the Array Studio software package version 10.1.2.9 (OmicSoft, Qiagen). Mapping of RNA-Seq reads (fastq files) to the human genome was done using Omicsoft gene model 20130723 with human B37.3 as reference genome and OSA4 as aligner. Genes with FPKM <1 were filtered out according to 50% fraction. Principal component analysis has been applied to all samples as a quality control assessment. Differentially expressed genes were detected by applying the DESeq2 statistical test to the comparisons of PRKAB1^{-/-} and PRKAB2^{-/-} clones *versus* WT. *P*-values were adjusted by the Benjamini-Hochberg procedure to control FDR. Changes in expression levels >1.3-fold and an adjusted *p*-value <0.05 were considered significant. Pathway and mechanistic analyses of differentially expressed genes were performed using Ingenuity Pathway Analysis (Qiagen).

Gene Ontology (GO) enrichment analysis

GO enrichment analysis was performed using an online tool (RRID:SCR_002811) being based on the PANTHER classification system. Datasets containing genes being differentially expressed between WT and PRKAB1^{-/-} or PRKAB2^{-/-} hiPSCs were subjected to the PANTHER Overrepresentation test for GO biological process. Release version of the GO database was 2019-10-08. Fisher's exact test with FDR correction was calculated automatically. Only results with FDR <0.05 were displayed.

qRT-PCR

RNA isolation was performed in a QIAcube device using an RNeasy kit (Qiagen). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcrip-

tion and qRT-PCR was performed with TaqMan[®] Assays (Life Technologies) in a LightCycler 480 instrument (Roche). Raw values were normalized to GAPDH. Assay IDs are listed in Table S1.

Western blotting

SDS-Page followed by Western blotting was performed by standard procedures. Antibodies were from Cell Signaling Technology. Recombinant AMPK protein (A2/B1/G2, A2/B2/G2; SignalChem) was plotted as a concentration curve and used to calculate the actual amount of AMPK β 1 and AMPK β 2 protein from total cell lysates.

Immunocytochemistry

For immunofluorescence staining, cells were detached and reseeded on laminin-coated 96-well plates (PerkinElmer). After attachment, cells were fixed in 4% PFA and staining was performed following a standard protocol. Primary antibodies were rabbit anti-cTnT (Abcam), mouse anti- α -actinin (Sigma), goat anti-Oct3/4 (R&D Systems). Alexa Fluor 488 or 555-conjugated secondary antibodies were used (Invitrogen) and nuclei were stained with Hoechst 33342 (Roche). Images were taken and analyzed using a spinning disc confocal microscope (PerkinElmer) and the corresponding imaging software Harmony V4.8.

Statistical analysis

Data are shown as mean \pm S.E. Statistical significance was analyzed by two-tailed Student's *t* test (GraphPad Prism software version 8.0.2 for Windows). *p* values <0.05 were considered significant. Maximal three significant digits are shown.

Data availability

Raw data of RNA-sequencing is available at NCBI-GEO under accession number GSE144043.

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Conflict of interest—At the time of manuscript preparation, all authors were employees of Sanofi-Aventis Deutschland GmbH.

Abbreviations—The abbreviations used are: AMPK, AMP-activated protein kinase; hiPSC, human induced pluripotent stem cells; ACC, acetyl-CoA carboxylase; miRNA, microRNA; gRNA, guide RNA;

GO, Gene Ontology; hESC, human embryonic stem cell; CM, cardiomyocyte.

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