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An Unbiased Proteomics Method to Assess the Maturation of Human Pluripotent Stem Cell-Derived Cardiomyocytes

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Abstract

Rationale: Human pluripotent stem cells-derived cardiomyocytes (hPSC-CMs) exhibit the properties of fetal CMs, which limits their applications. Various methods have been used to promote maturation of hPSC-CMs; however, there is a lack of an unbiased and comprehensive method for accurate assessment of the maturity of hPSC-CMs.

Objective: We aim to develop an unbiased proteomics strategy integrating high-throughput top-down targeted proteomics and bottom-up global proteomics for the accurate and comprehensive assessment of hPSC-CM maturation.

Methods and Results: Utilizing hPSC-CMs from early- and late-stage two-dimensional monolayer culture and three-dimensional engineered cardiac tissue, we demonstrated the high

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AUTHOR CONTRIBUTION

W.C., J.Z., W.J.dL., Z.R.G., J.C.R., T.J.K. and Y.G. designed research; W.C., J.Z., W.J.dL., Z.R.G., H.K., E.T.F., Z.L. T.T., S. D. M. and M.G. performed research; W.C., J.Z., W.J.dL., Z.R.G., H.K., E.T.F., T.T., S. D. M., M.B., S.M., J.C.R., T.K.J. and Y.G. analyzed data; W.C., J.Z., W.J. dL., Z.R.G., H.K., J.C.R., T.J.K., and Y.G. wrote the manuscript.

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reproducibility and reliability of a top-down proteomics method, which enabled simultaneous quantification of contractile protein isoform expression and associated post-translational modifications (PTMs). This method allowed for the detection of known maturation-associated contractile protein alterations and, for the first time, identified contractile protein PTMs as promising new markers of hPSC-CMs maturation. Most notably, decreased phosphorylation of α-tropomyosin was found to be associated with hPSC-CM maturation. By employing a bottom-up global proteomics strategy, we identified candidate maturation-associated markers important for sarcomere organization, cardiac excitability, and Ca^{2+} homeostasis. In particular, up-regulation of myomesin-1 and transmembrane 65 were associated with hPSC-CM maturation and validated in cardiac development, making these promising markers for assessing maturity of hPSC-CMs. We have further validated α-actinin isoforms, phospholamban, dystrophin, αB-crystallin and calsequestrin 2 as novel maturation-associated markers, in the developing mouse cardiac ventricles.

Conclusions: We established an unbiased proteomics method that can provide accurate and specific assessment of the maturity of hPSC-CMs, and identified new markers of maturation. Furthermore, this integrated proteomics strategy laid a strong foundation for uncovering the molecular pathways involved in cardiac development and disease using hPSC-CMs.

Keywords

human pluripotent stem cells; cardiovascular proteomics; top-down mass spectrometry; quantitative proteomics; maturation; Proteomics; Stem Cells; Basic Science Research

INTRODUCTION

Directed differentiation of human pluripotent stem cells (hPSCs) into cardiomyocytes (CMs) provides an unprecedented opportunity for patient-specific cardiac disease modeling, drug discovery, cardiotoxicity screening, and cell-based therapy development.[1–4] However, hPSC-CMs exhibit the morphological, electrophysiological, metabolic, and contractile properties of fetal CMs, which limit their applications.[1, 5, 6] Promoting hPSC-CMs to acquire the key features of adult CMs is critical to realize the full promise of hPSC-CM technology. Consequently, significant effort has been dedicated to promoting maturation of hPSC-CMs, including biochemical, mechanical, and electric interventions, as well as tissue engineering.[5–8] Traditionally, cell morphology, contractile function, electrophysiological properties, and changes in protein expression have been used as indicators of hPSC-CM maturation.[5] However, there remains no consensus regarding which markers are most suitable for accurate assessment of hPSC-CM maturity.[6] Unfortunately, some of the frequently used markers are not unique to hPSC-CMs, and the expression of others are highly variable and not always indicative of maturation.[6] Moreover, a number of existing markers are highly dependent on the measurement conditions, and some may provide a false assessment if considered as stand-alone indicators of maturation.[6] Hence, there is an urgent need to establish an unbiased method for the accurate and specific assessment of hPSC-CM maturity.

Discovery-based “omics” technologies have revolutionized research by enabling large-scale analysis of the molecular constituents of biological systems without *a priori* knowledge and,

thus, can provide an unbiased method to assess hPSC-CM maturity.[9] Although transcriptome analysis of hPSC-CMs yields information regarding the differential regulation of genes at the mRNA level,[10] transcript levels by themselves are not sufficient to predict protein levels.[11] Moreover, transcript analysis offers no information regarding protein post-translational modifications (PTMs), which play essential roles in the regulation of protein activity and function.[12, 13] Therefore, direct interrogation of the proteome is necessary to provide definitive evidence of differential expression of protein isoforms and changes in their PTMs. Conventional mass spectrometry (MS)-based bottom-up proteomics (analysis of peptides digested from proteins) is a powerful method for the rapid and large-scale determination of differential regulation of thousands of proteins.[13] However, bottom-up proteomics is suboptimal for detecting changes in protein isoforms, especially those with high sequence homology and protein PTMs, due to difficulties in achieving full sequence coverage.[14] In contrast, despite relatively limited proteome coverage, top-down MS-based proteomics (analysis of intact proteins without proteolytic digestion) is the most powerful technology for quantitatively assessing diverse protein PTMs and sequence variants simultaneously in one spectrum.[12, 15]

Here, we present an unbiased MS-based proteomics method integrating top-down targeted proteomics and high-throughput bottom-up global proteomics for the accurate and comprehensive assessment of hPSC-CM maturation. We demonstrate high reproducibility and reliability of the targeted top-down method for concurrent quantification of sarcomeric (contractile) protein isoforms and PTMs. Subsequently, this top-down method was applied to the analysis of hPSC-CMs from early- and late-stage two-dimensional (2D) monolayer culture, as well as hPSC-CMs cultured in three-dimensional (3D) engineered cardiac tissue (ECT) constructs,[16, 17] for the assessment of hPSC-CM maturation. Moreover, we utilized bottom-up quantitative proteomics to assess changes in protein expression globally during hPSC-CM maturation. This integrated proteomics platform not only enabled the detection of molecular changes previously known to occur during hPSC-CM maturation, but also facilitated the discovery of novel candidate markers of maturation, including alterations in key contractile protein PTMs and the expression of other non-contractile molecules involved in sarcomere organization, cardiac excitability, and Ca^{2+} homeostasis. Subsequent validation of the identified changes in the ventricular myocardium of mice during development provided evidence that these alterations are *bona fide* markers of hPSC-CM maturation.

METHODS

Full details of the reagents and experimental procedures are described in the Online Supplement. All proteomics data can be accessed through ftp://massive.ucsd.edu/MSV000082985.

hPSC culture and CM differentiation.

One human induced pluripotent stem cell (hiPSC) line, DF19–9-11T, and one human embryonic stem cell (hESC) line, H1, were used in this study. The hiPSC line DF19–9-11T was previously derived from human foreskin fibroblasts using non-integration episomal vector-mediated reprogramming with six factors *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-Myc*,

KLF4 and *SV40LT* as described by Yu et. al.[18] hiPSCs and hESCs were maintained on Matrigel (GFR, BD Biosciences)-coated 6-well plates in mTeSR1 medium. Cell lines were between passage 30 and 75. hiPSCs and hESCs were differentiated into CMs using a small molecule-directed protocol as described previously.[3, 4, 19]

ECT preparation.

See the Online Supplement for detailed descriptions of all experiments performed with ECTs. CMs were differentiated from DF19–9-11T hiPSCs using the small molecule protocol as described above.[3, 4, 19] At day 30 of differentiation without lactate selection, cells were dissociated with TrypLE and subsequently suspended in 20 mL ECT medium and incubated to form small and uniform clusters of viable cells. Approximately 2×10^6 hiPSC-CMs were mixed with 1.25 mg/mL fibrinogen and 0.5 unit of thrombin in 200 μL ECT medium to prepare the ECT constructs. Following polymerization of the fibrin matrix, ECT were fed with ECT media and cultured for 14 to 44 days. Assessment of ECT function and histology were performed as previously described.[17]

Top-down MS analysis and quantification of sarcomeric protein isoform and PTMs.

Sarcomeric proteins were enriched from freshly isolated hPSC-CMs from monolayer or ECT culture. Liquid chromatography (LC)-MS analysis was carried out using a NanoAcquity Ultra-high Pressure LC system (Waters) coupled to a high-resolution Impact II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker Daltonics). Mass spectra were collected at a scan rate of 0.5 Hz over the 500–2000 *m/z* range. All top-down LC-MS data were processed and analyzed using the DataAnalysis v4.3 for protein quantification. For a specific protein isoform, the most abundant 3–5 charge state ions were selected for generating an extracted ion chromatogram (EIC). The area under curve (AUC) of the EIC of a specific protein isoform represents its abundance. Relative quantification of protein PTMs were performed as reported previously.[20–22] All comparisons drawn between early- and late-stage hPSC-CMs were from the same differentiation batch.

Global label-free quantitative proteomics analysis.

Detailed sample preparation methods can be found in the Online Supplement. The digested peptides were loaded onto a C18 trap for desalting and then separated on a 250 mm PicoFrit C18 capillary column. The capillary column tip was customized to fit into the CaptiveSpray nanoESI source (Bruker Daltonics). The most abundant 30 ions were selected for fragmentation by tandem MS (MS/MS). Protein identification (with at least 1 unique peptide) and quantification was performed using the MaxQuant v1.5.7.[23, 24] The Welch's modified *t*-test (two-tailed) was applied to evaluate the statistical significance of change. Proteins were considered significantly up- or down-regulated with a *p*-value smaller than 0.01, and a more than 1.4-fold change in the expression level.

RESULTS

hPSCs, including both hiPSCs and hESCs, were differentiated into CMs using a small molecule-directed differentiation protocol,[3, 19] which produced hPSC-CMs predominantly (91.5%) of the ventricular lineage based on electrophysiological properties.[19, 25] To establish

a quantitative platform for assessment of hPSC-CM maturation, we first employed a simple maturation method using extended culture in 2D monolayer and avoided adding growth factors or signaling molecules used in some powerful maturation protocols[26, 27] that may result in proteome alterations that are independent of the maturation process (Fig. 1A). Following extended culture, hPSC-CMs exhibit increased cell size, greater myofibril density and organization, and improved contractile function.[28] Additionally, to assess the role of 3D-culture on hPSC-CM maturation, hPSC-CMs at Day 30 post-differentiation were dissociated for the preparation of ECTs, and the ECTs were collected at early and late stage accordingly.

The hPSC-CMs from 2D monolayer culture and 3D ECT were used for top-down targeted proteomics analysis with a focus on the analysis of sarcomeric proteins (the contractile proteins) (Fig. 1B). Studies have shown that multiple sarcomeric protein isoforms undergo a fetal-to-adult transition during cardiac development,[29–31] including switching from α- to β-myosin heavy chain (α-MHC, β-MHC), slow skeletal to cardiac troponin I (ssTnI, cTnI), and the atrial isoforms of myosin light chain 1 and 2 (MLC1a, MLC2a) to the corresponding ventricular isoforms (MLC1v, MLC2v) (Fig. 1B).[29–31] The ability to detect these changes was used as a means of validating the proteomics method for tracking the maturity of hPSC-CMs. Additionally, this top-down proteomics method allowed for the identification of new candidate maturation markers in the contractile apparatus, such as myofilament protein PTMs. Maturation of hPSC-CMs is known to impact the expression of genes related to metabolism, excitability, Ca^{2+} cycling, and cell cycle, which all change during development.[6] To further provide an unbiased analysis for the differential regulation of global protein expression beyond the sarcomeres, we employed a label-free bottom-up quantitative proteomics method to identify molecular alterations between hPSC-CMs from early- and late-stage cultures (Fig. 1C).

Assessment of Reproducibility and Robustness of Top-down Proteomics for Simultaneous Quantification of Isoforms and their PTMs

To accurately quantify protein isoform and PTM changes for the assessment of hPSC-CM maturation, we developed a robust liquid chromatography (LC)-MS-based top-down proteomics platform for targeted analysis of sarcomeric protein isoforms and their PTMs. We first evaluated the reproducibility of contractile protein enrichment from hPSC-CMs at Day 30. The cell pellets were first homogenized in a gentle neutral buffer (without detergent) to extract soluble (predominantly cytosolic) proteins and preserve the integrity of the sarcomere, followed by further homogenization in an acidic and MS-compatible solution for extracting the sarcomeric proteins.[21, 32] This extraction method has proven highly reproducible for hiPSC-CMs (DF19–9-11T line) from early- and late-stage cultures (Fig. 2A). MHC was highly enriched in the sarcomeric protein fraction, and immunoblotting showed that ssTnI, the troponin I isoform expressed in immature/fetal CMs, was present exclusively in the sarcomeric protein fraction (Fig. 2A, B).

After confirming reproducible protein separation and mass spectrometer response (Supplementary Fig. I), we examined the linearity of the instrument response by injecting a defined protein quantity (100, 200, 300, 500, 750 ng) from the sarcomeric protein fraction (Fig. 2C). Quantification of the sarcomeric protein was performed by generating extracted

ion chromatogram (EIC) specific to each protein, and protein expression was represented as the area under curve (AUC) (Supplementary Fig. II). From a single top-down LC-MS run, the EICs of the major sarcomeric proteins (< 50 kDa), including cardiac troponin T splicing isoform 6 (cTnT6, an adult isoform of cTnT, Supplementary Fig. III), ssTnI, α-tropomyosin (α-Tpm, formally known as Tpm2.2st[33]), MLC1v, MLC1a, MLC2v, MLC2a, and cardiac α-actin (cα-actin), were generated and the abundance of each protein is represented by the AUC (Fig. 2C). cTnI remained undetected in the extract from hiPSC-CMs at Day 30, either because cTnI was not expressed or expressed below the detection limit of the instrument (Fig. 2C). A complete list of proteins identified by the top-down proteomics can be found in Supplementary Table I and Supplementary Fig. IV. We further confirmed the linearity of the instrument response as demonstrated by the linear correlation of the AUCs of MLC1v and MLC1a with varied total sarcomeric protein (100–750 ng) injection (Fig. 2D), and the injection replicates proved highly consistent with less than 8% coefficient of variation (Fig. 2E). The linear curves of the major sarcomeric proteins (< 50 kDa), including the above-mentioned proteins and cTnT splicing isoform 1 (cTnT1, a fetal isoform of cTnT, Supplementary Fig. III), are shown in Fig. 2F, and the mutual linear range of all major sarcomeric proteins is between 100 and 500 ng proteins from the sarcomeric protein extracts.

These results demonstrated reliability of the quantification method given equal amount of total proteins were injected, and quantification was performed over the linear range. To further demonstrate the reliability of this method for the quantification of sarcomeric protein isoforms as markers of hPSC-CM maturation, we tested CMs differentiated from a different pluripotent stem cell line, a human embryonic stem cell (hESC) H1 line. This method has also proven highly robust and quantitative for the assessment of sarcomeric isoform expression in hESC-CMs (Supplementary Fig. V).

Changes in Sarcomeric Protein Isoforms Following Prolonged Culture of hPSC-CMs Revealed by Top-down Proteomics

Following the validation of the reproducibility and quantitative capabilities of the top-down LC-MS-based proteomics method, we evaluated whether this method permits reliable determination of hPSC-CM maturity. The expression of MLC2v and MLC2a is a commonly used indicator to determine the maturity of hPSC-CMs.[5] As expected, the expression of MLC2v increased whereas that of MLC2a decreased in hESC-CMs with prolonged culture by immunostaining (Fig. 3A). After ensuring that the purity of hESC-CMs from the early- and late-stage cultures was greater than 90% (Fig. 3B), we first confirmed that prolonged 2D monolayer culture promotes hESC-CM maturation by metabolically profiling cells cultured for approximately 30 days compared to 60 days. The rates of oxygen consumption (OCR) and extracellular acidification (ECAR) were measured using the Seahorse XF-24 metabolic flux analyzer. As cardiomyocytes mature, there is a switch from primarily glycolytic to oxidative metabolism. Although baseline OCR did not differ between these cells, OCR was significantly increased in Day 60 compared to Day 30 hPSC-CMs following addition of carbonyl cyanide-4 phenylhydrazone/oligomycin (FCCP/Oligo), an uncoupling agent (Fig. 3C). Moreover, at baseline, ECAR was significantly lower in Day 60 versus Day 30 cells, which is consistent with a lower basal rate of glycolysis, even though ECAR did not differ

between these cells following FCCP/Oligo addition (Fig. 3D). These data are consistent with a more mature metabolic phenotype in Day 60 compared to Day 30 hPSC-CMs.

Next, we analyzed the hESC-CMs by top-down LC-MS to compare with the non-failing human adult left ventricular (LV) tissue (Fig. 3E, F). The majority (greater than 90%) of hESC-CMs derived from the small molecule protocol are fated to the ventricular lineage.[25] but hESC-CMs from both early- and late-stage cultures exhibit significant levels of expression of both the ventricular and atrial isoforms, indicating immaturity of the ventricular hESC-CMs from the 2D culture, compared to the left ventricular tissues. MLC2v, a common marker for evaluating the maturity of hPSC-CMs, increased significantly with extended culture, along with a decrease in the MLC2a expression (Fig. 3G). Intriguingly, the expression of both MLC1a and MLC1v decreased in the hESC-CMs with prolonged culture (Fig. 3G). Compared to the mature human LV and LA tissue, wherein cTnI was expressed robustly with undetectable ssTnI (Supplementary Fig. VI), hESC-CMs from both the early- and late-stage culture were considered fetal-like due to undetectable cTnI and robust expression of ssTnI (Fig. 3F). Nevertheless, hESC-CMs at Day 60 exhibited increased maturity as measured by decreased cTnT1 (the fetal cTnT isoform) (Fig. 3G). cTnT6, the major adult cTnT isoform expressed in cardiac ventricular tissue[34, 35] remained unchanged (Supplementary Fig. VII).

Comparable results were observed using CMs differentiated from the human iPSC line, DF19–9-11T. All sarcomeric protein isoforms detected show consistent changes in expression between cells from early- and late-stage cultures, except for the expression of MLC1a (Fig. 3G, Supplementary Fig. VIII) which showed an increase in expression at day 59 in the hiPSC-CMs in contrast to the decrease in hESC-CMs. The different findings for MLC1a may result from different hPSC lines such that the degree of maturation is different between the lines at the time points studied with complex developmental dynamics of MLC1a expression or from a varied proportion of ventricular and atrial cells within the hPSC-CM population studied.

Sarcomeric Protein PTMs as Potential Markers for hPSC-CM Maturation

The LC-MS-based top-down proteomics method we developed not only allows for the quantification of protein expression across multiple samples, but also permits simultaneous relative quantification of protein PTMs. From the same top-down LC-MS datasets wherein protein expression data were extracted, high-resolution mass spectra were examined for the quantification of protein PTMs. Phosphorylation of the cTnT isoforms was observed in the hESC-CMs similarly as in the non-failing human adult LV tissue (Fig. 4A). ssTnI in the hESC-CMs was also found to be phosphorylated as indicated by a species with a mass increase of 80 Da (Fig. 4B). In addition, a potentially novel modification with an increase of 248 Da relative to the mass of ssTnI was detected (Fig. 4B); however, ssTnI was not detected in the human LV tissue. In contrast, cTnI was found only in the mature LV tissue with two phosphorylated species detected (Fig. 4C).

In the non-failing human adult LV tissue, mono-phosphorylated α-Tpm was low (approximately 8%) relative to the un-modified α-Tpm,[36] whereas the levels of α-Tpm phosphorylation were significantly higher in the hESC-CMs (Fig. 4D). However, extended

culture lead to decreased α-Tpm phosphorylation in the Day 60 hESC-CMs compared to the Day 31 cells, consistent with the Day 60 cells being relatively more mature (Fig. 4D). Furthermore, a previous study demonstrated a decline of α-Tpm phosphorylation during cardiac development in rat hearts.[37] Collectively, phosphorylation of α-Tpm may also serve as a validated marker of hPSC-CM maturation. To the best of our knowledge, this is the first study suggesting protein PTMs can be used as markers of hPSC-CM maturation.

Though MLC2a was not detected in the non-failing human LV tissue, the phosphorylation of MLC2a decreased in the Day 60 hESC-CMs compared to Day 31 cells (Fig. 4E). Similarly, MLC2v phosphorylation was decreased and was nearly abolished in the hESC-CMs following extended culture; however, in non-failing human LV tissue, MLC2v phosphorylation was detected at a low level (Fig. 4F). In addition to thin and thick filament proteins, we identified phosphorylated forms of muscle LIM protein (MLP) and cysteine-rich protein 2 (CRIP2) in the hESC-CMs, similarly as seen in the human tissue (Fig. 4G, H, Supplementary Fig. IX). MLP is an important Z-disc protein specifically expressed in cardiac and skeletal muscles, and mutations in MLP are known to be associated with dilated and hypertrophic cardiomyopathies.[38] Although a phosphorylated form of MLP was detected in hESC-CMs, the level of MLP phosphorylation appeared to be lower than that in non-failing human adult LV tissue (Fig. 4G). Interestingly, cysteine-rich protein 2 (CRIP2) is highly expressed in the heart during development and in the adult heart,[39, 40] and was previously identified as a heart vascular marker,[40] but this study confirmed the expression of CRIP2 in the CMs (Fig. 4H). Additionally, we have, for the first time, detected phosphorylation of CRIP2 in the hESC-CMs and human heart tissue, although the level of phosphorylation was lower than that observed in adult heart tissue (Fig. 4H).

Quantification of the sarcomeric protein PTMs is shown in Fig. 4I. Significant changes were observed in the phosphorylation of ssTnI, cTnT, α-Tpm, MLC2a, and MLC2v in Day 60 hESC-CMs compared to Day 31 cells (Fig. 4I). Since ssTnI and MLC2a were not detected in the non-failing adult LV tissue, the phosphorylation of ssTnI and MLC2a may not be good markers of hPSC-CM maturation at the later stage of development (beyond Day 60). On the other hand, since α-Tpm is expressed throughout hPSC-CM development and in adult hearts,[37] and since α-Tpm phosphorylation has been consistently demonstrated to decline in the developing rat hearts,[37] the declining phosphorylation of α-Tpm observed in hPSC-CM's can serve as a novel marker of maturation.

Global Differential Regulation of Proteins in hPSC-CM Maturation

Although the top-down LC-MS-based quantitative proteomics has proven to be a robust method for quantifying highly homologous protein isoforms concurrently with protein PTMs, detection and quantification of high molecular weight (MW) proteins and low abundance proteins from the hPSC-CMs remains challenging due to an exponential decay of signal-to-noise ratio with increasing MW.[12, 41] Therefore, to investigate the differential regulation of hPSC-CM protein expression beyond the contractile proteins in early- and late-stage cultures, we undertook a label-free bottom-up proteomics approach for global quantitative profiling of protein expressions in the hPSC-CMs.

hiPSC-CMs from early- and late-stage culture with comparable CM purity (Supplementary Fig. X) were harvested and analyzed using an unbiased label-free quantitative proteomics approach, allowing for confident identification of 1898 protein groups at Day 30 and 59, among which 152 and 114 proteins were found to be significantly ($p < 0.01$, > 1.4-fold change) up- and down-regulated, respectively (Fig. 5A, Supplementary Table II). To first validate the label-free quantification method for assessing the maturation of hiPSC-CMs, we surveyed a number of proteins that are known to be regulated developmentally, or are critical to contractile function and Ca^{2+} handling in relatively mature CMs. In a panel of 27 proteins, we have observed significant up-regulation of β-MHC and down-regulation of α-MHC (Fig. 5B), which is consistent with a previous study that suggested a significant decrease in α-MHC expression in the ventricles of the human hearts between 7 and 12 weeks of gestation.[29] In addition, we identified a number of high MW sarcomeric proteins that were significantly up-regulated in Day 59 hiPSC-CMs compared to the Day 30 cells, including titin, α-actinin 2 (sarcomeric-specific isoform), cardiac myosin binding protein C (cMyBP-C) and myomesin 1 (MYOM1) (Fig. 5B). Myomesin is an important structural component of the M-line of the sarcomeres, and is essential for the elasticity of the contractile apparatus.[42] The formation of M-lines is considered indicative of sarcomeric structural maturation,[43, 44] and is consistent with increased myomesin expression in hPSC-CMs following prolonged culture. Of the 266 proteins that were differentially regulated (Fig. 5B), we identified 8 surface membrane proteins, including fatty acid translocase (CD36), transmembrane protein 65 (TMEM65) and transferrin receptor protein 1 (CD71), which were up-regulated with hPSC-CM maturation (Supplementary Table III).

Subsequently, we performed protein interaction analysis and categorized the differentially regulated proteins based on their biological function. We found each category contains proteins from both up- and down-regulated protein groups, but the proteins involved in metabolic processes, cellular energy production, and oxidation-reduction processes were predominantly up-regulated (Fig. 5C). This suggests that hiPSC-CMs were undergoing major metabolic changes between Day 30 to 59 of culture, which may reflect a change from glycolytic predominant metabolism in the early heart to fatty acid β-oxidation in post-natal heart.[45] In particular, there was a decrease in enolase 1 (ENO1), a glycolytic enzyme known to be down-regulated during cardiac development,[46] and up-regulation of acyl-coA dehydrogenase family member 9 (ACAD9), acetyl-CoA acetyltransferase (ACAT1), fatty acid translocase (CD36), and carnitine palmitoyltransferase 2 (CPT2) (Fig. 5C, Supplementary Table II), which are important enzymes in β-oxidation of fatty acids. However, the changes in metabolic enzymes were complex with expression of some glycolytic enzymes increasing such as muscle phosphofructokinase (PFKM) and at least one enzyme associated with β-oxidation of fatty acid, malonyl-CoA decarboxylase (MLYCD), decreasing in expression (Fig. 5C, Supplementary Table II).

A detailed analysis of the sub-interactome of proteins involved in or actin-myosin interaction revealed that early hiPSC-CMs expressed myosin light chain 6 (MYL6), myosin heavy chain 9 and 10 (MYH9, MYH10), the motor protein isoforms expressed in smooth muscle or non-muscle cells, which were down-regulated following prolonged culture (Fig. 5D). We found robust expression of both α-actinin 1 and α-actinin 2, and the expression of α-actinin 1 decreased whereas the expression of α-actinin 2 increased in the hiPSC-CMs with

prolonged culture (Fig. 5D). α-actinin 2 is the predominant isoform expressed in the adult cardiac muscle, whereas α-actinin 1 is generally considered as the non-sarcomeric isoform expressed in non-muscle cells.[47, 48] These results indicated expression of both α-actinin 1 and α-actinin 2 in the early immature CMs, with an increase in α-actinin 2 with maturation. The interactome analysis revealed a wide variety of proteins interacting with the α-actinin isoforms (Fig. 5D), suggesting a key role for α-actinin in CM development and, possibly, maturation.

Among the up-regulated proteins that interact with the sarcomeric proteins, many are involved in cell adhesion and cell junction assembly including dystrophin (Fig. 5D), an important component of the sarcolemma membrane complex.[49] In addition, proteins involved in cardiac excitability including α1-syntrophin were found to be up-regulated (Fig. 5D). α1-syntrophin has been shown to regulate CM membrane action potential via regulation of the cardiac sodium channels,[50, 51] and may contribute to the increase in voltage-gated sodium current observed with maturation. The maturation of hPSC-CMs requires orchestrated protein synthesis and folding and, consistently, we observed up-regulation of a number of molecular chaperones/co-chaperones, including αB-crystallin (Fig. 5A, C), a small heat shock protein recently shown to play key roles in the regulation of cardiac hypertrophy[52] and maintenance of mitochondrial homeostasis in CMs[53]. Moreover, we found that both phospholamban (PLB), a regulator of the cardiac sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA), and calsequestrin (CASQ2), a SR Ca^{2+} binding protein, were identified in Day 59 hiPSC-CMs samples, but not in Day 30 cell samples (Supplementary Table II).

For the proteins identified to be up- or down-regulated in the hiPSC-CMs following prolonged culture, we further examined their expression pattern during developmental using embryonic and post-natal mouse hearts given the inability to obtain reproducible timed human embryonic and post-natal human cardiac tissue. The mouse hearts were harvested at embryonic day 15 (E 15), post-natal day (PND) 1, 10, 21, and at adulthood (3-month old) (Fig. 5E, F). The ventricles were separated from the atria, and the ventricular tissues were used for analysis because the vast majority of hPSC-CMs were of the ventricular lineage. Immunoblotting showed increased expression of dystrophin, α-actinin 2, cTnI, αB-crystallin, PLB, and CASQ2, with a progressive decrease in the expression of α-actinin 1, in the developing mouse ventricles (Fig. 5E, F, Supplementary Fig. XI), which were consistent with the observation in hPSC-CM maturation. These results demonstrate the great promise of these proteins as new markers of hPSC-CMs at various stages of development, and the power of label-free quantitative proteomics for identifying novel markers of hPSC-CM maturation.

As expected, changes of some sarcomeric proteins were not consistent between the bottom-up and the top-down approaches. For example, MLC2a, which showed significant down-regulation by the top-down approach (Fig. 3G), did not appear to change significantly in the bottom-up analysis (Fig. 5B). One explanation for this is that in the bottom-up approach, proteins were quantified relative to the total proteins in the whole cell lysates, whereas in the top-down approach, protein quantification was performed relative to the proteins in the sarcomere-enriched fraction. Since sarcomere contents continue to increase in the process of

CM maturation,[43, 54] it is likely that a protein, of which the expression decreases relative to the sarcomeres, may appear unchanged or even increased when compared to total protein contents in the cells.

Top-down LC-MS Analysis of CMs Cultured in 3D ECT

Recent development of tissue engineering technology has enabled the construction of ECTs, wherein CMs are cultured in a 3D environment with extracellular matrix to support the contacts between CMs and improve CM function *in vitro*.[6, 16, 55, 56] Previous studies have implicated improved hPSC-CM maturation with ECT culture compared to conventional 2D monolayer culture.[55] However, a detailed analysis of contractile protein isoform expression and their PTMs in hPSC-CMs cultured in 3D ECT has not been performed. Therefore, we employed the top-down LC-MS method to quantify the expression and PTMs of sarcomeric proteins in the ECT CMs as compared to the cells from 2D monolayer.

CMs were differentiated from DF19–9-11T hiPSCs in monolayer for 30 days, after which cells were dissociated and seeded into a fibrin-based matrix to form the 3D ECT constructs. ECTs were cultured for an additional 14–44 days. The hematoxylin and eosin stain showed a uniform distribution of hiPSC-CMs throughout the matrix (Fig. 6A). Immunostaining of the ECT showed localization of α-actinin and cMyBP-C to the highly organized sarcomeres, as well as longitudinally orientated hiPSC-CMs within the ECT (Fig. 6A). To confirm that extended 3D culture promotes hiPSC-CM maturation, twitch force and Ca^{2+} transients were recorded in early- (Day 30) and late-stage (Day 60) ECTs (Fig. 6B,C). While peak twitch force amplitude was not different between early- and late-stage ECTs, the time to peak twitch force (CT_{100}) and time to 50% twitch force decay (RT_{50}) were significantly decreased in late-stage ECTs (Fig. 6D). Furthermore, the time to peak cytosolic Ca^{2+} ($Ca^{2+}T_{100}$) was significantly decreased in late-stage compared to early-stage ECTs (Fig. 6E). Collectively, these results suggest that extended 3D culture in ECTs enhances the maturity of hiPSC-CMs by promoting efficient coupling of between intracellular Ca^{2+} entry and release.[57, 58] Furthermore, sarcomeric proteins were reproducibly extracted and detected by top-down MS from 3-week ECTs (Supplementary Fig. XII).

Next, we performed parallel analysis on hiPSC-CMs from the same batch of differentiation cultured in either 2D or 3D cultures for the same length of culture (Supplementary Fig. XIII). Due to interference from the matrix proteins in ECTs, quantification of sarcomeric protein isoforms cannot be performed by directly comparing the AUCs of the same protein across different samples under the current conditions and, therefore, the relative expression of the adult to fetal isoforms were compared when the hiPSC-CMs from 2D and 3D cultures were analyzed. At Day 76 post-differentiation, cTnI, the adult isoform, can be detected in the hiPSC-CMs with low abundance, whereas ssTnI, the fetal isoform, remains robustly expressed (Fig. 6F). The expression of cTnI relative to ssTnI was higher in the hiPSC-CMs from the 3D culture compared to the cells maintained in the 2D culture (Fig. 6F). The increased expression of cTnI relative to ssTnI in the 3D hiPSC-CMs compared to the 2D cells at the late-stage culture provided molecular evidence that 3D ECT can promote hiPSC-CM maturation.[6] We also observed a reduction in the phosphorylation of α-Tpm in the 3D-cultured cells as compared to those cultured in monolayer (Supplementary Fig. XIII), which

is consistent with improved maturation with prolonged culture. This further supports that 3D culturing may promote maturation of hiPSC-CMs, and that α-Tpm phosphorylation might serve as a novel marker for hPSC-CM maturation. Nevertheless, hiPSC-CMs from the 3D ECTs remain relatively immature compared to non-failing adult LV tissues, given the strong expression of ssTnI.

For the myosin light chain isoforms, prolonged culture duration rather than the culture matrix (2D vs. 3D) had a greater impact on the expression of MLC1v relative to MLC1a (Fig. 6G) and the expression of MLC2v relative to MLC2a (Fig. 6G). In particular, the expression of MLC1v relative to MLC1a decreased in the 2D CMs following prolonged culture (Fig. 6G), which is consistent with Fig. 3F. This may be due to the cells still being in the early stage of development wherein MLC1a expression was still increasing before it reached maximum and started to decrease. The expression of MLC2v relative to MLC2a was not significantly altered between the cells from the 3D and 2D culture (Fig. 6H). Since the expression of MLC2v relative to MLC2a was already high (Fig. 6H), minor changes due to the altering of the culturing method may not have a significantly detectable impact on the expression of MLC2v relative to MLC2a.

DISCUSSION

Despite substantial interest in hPSC-CM in basic and translational research, the immaturity of hPSC-CMs poses significant challenges, particularly for disease modeling, cell therapy development, and drug screening. Promoting hPSC-CM maturation requires accurate assessment at the molecular level. To facilitate the analysis of the maturity of hPSC-CMs, we developed an unbiased proteomics strategy combining the strengths of both bottom-up and top-down proteomics to assess hPSC-CM maturity. With the bottom-up proteomics method providing global identification and quantification of proteins in-breadth, and the top-down proteomics method offering in-depth analysis of protein isoform expression and their PTMs, we have demonstrated the power of an integrated proteomics strategy for the comprehensive characterization of hPSC-CM maturation.

For the first time, we developed a quantitative top-down proteomics method that can provide concurrent quantification of protein isoform expression and PTMs with high reproducibility and throughput (Fig. 2–4). The entire process of top-down analysis, including sample preparation, takes less than 2 hrs. Compared to the traditional immunoblotting technique, with a single 40 min LC-MS run, we were able to detect and quantify the expression level of the major sarcomeric proteins including cTnT, cTnI/ssTnI, α-Tpm, MLC1v/MLC1a, MLC2v/MLC2a, cα-actin (Fig. 3, Supplementary Table I) and their PTMs, most notably phosphorylation (Fig. 4). Supported by the quantitative top-down proteomics method, we can perform a direct assessment of the hPSC-CM maturity compared to that of the adult CMs from non-failing human LV tissue (Fig. 3, 4). Overall, hPSC-CMs from both early- and late-stage 2D and 3D culture exhibit robust expression of ssTnI and extremely low expression of cTnI, consistent with all preparations being immature relative to adult LV CMs.[30, 59] Robust expression of MLC1a and MLC2a also indicated fetal-like phenotypes of hPSC-CMs, despite their being predominantly of the ventricular lineage. Our study confirms that MLC2v-to-MLC2a switch represents a relatively early maturation marker, whereas

ssTnI-to-cTnI switch occurs significantly later, and thus represents a later marker of hPSC-CM maturation.[60] These results demonstrated that the timing at which different contractile proteins switch isoforms during development is different, highlighting the importance of utilizing a panel of markers to accurately determine the maturation status of hPSC-CMs.

PTMs mediate protein activity and function, and are increasingly recognized as important regulators in numerous cellular processes.[61] Hence, protein PTMs may serve as important markers of hPSC-CM maturation. Notably, for the first time, we have identified decreasing phosphorylation of α-Tpm, a critical thin-filament regulating protein,[36] as a candidate maturation marker. There is a significant decrease of α-Tpm phosphorylation in hPSC-CMs following prolonged culture, and α-Tpm phosphorylation was found to be extremely low (less than 8%) in adult CMs (Fig. 4D, I). We have also observed that compared to the 2D cells, hPSC-CMs from 3D ECT exhibited a significant lower level of α-Tpm phosphorylation (Supplementary Fig. XIII) that was close to the level observed in the adult LV tissues, further supporting the promotion effects of 3D culture on hPSC-CM maturation. α-Tpm is expressed throughout hPSC-CM differentiation and maturation and in the adult hearts, and a previous study has validated the declined phosphorylation of α-Tpm in developing rat hearts.[37] Hence, phosphorylation of α-Tpm can also serve as a validated marker of hPSC-CM maturation.

Analysis of low abundance high MW proteins larger than 50 kDa remains a significant challenge in the field of top-down proteomics.[12, 41, 62] To address this, we employed a bottom-up global proteomics approach to complement the top-down analysis. The bottom-up proteomics approach outperformed the top-down method in the breadth of the proteome coverage, and permits the analysis of the high MW proteins, including the MHC isoforms, titin, and cMyBP-C, as well as low abundance proteins beyond the contractile apparatus, such as SERCA and ryanodine receptors (RYRs) (Fig. 5). Empowered by the global proteomics method, we identified the expression changes of large proteins in hPSC-CMs following prolonged culture, such as up-regulation of β-MHC, cMyBP-C and titin, and down-regulation of α-MHC (Fig. 5B). Previous studies suggest that human ventricular CMs switch from expressing the fast α-MHC isoform to the slow β-MHC during cardiac development[29] with approximately 90% β-MHC isoforms expressed in the non-failing human ventricles.[63] However, the expression of α- and β-MHC can be affected by pathological stress and heart failure, thus, compromising its reliability as a maturation marker in the setting of disease.[5] Myomesin is an important component of the sacomere M-lines,[42] and was also found up-regulated in hPSC-CMs following prolonged culture (Fig. 5). Myomesin plays critical role in the passive elasticity of the contractile apparatus, which is essential for increased force production with CM maturation. A prior study in chicken hearts also showed a continuing increase in the expression of myomesin at the protein level during development.[64] Thus, myomesin-1 a promising novel marker for hPSC-CM maturation.

The global label-free proteomics method also revealed that proteins involved in metabolic processes were predominantly up-regulated in hPSC-CMs following prolonged culture (Fig. 5C, Supplementary Table II). In particular, proteins responsible for fatty acid β-oxidation (e.g. ACAD9, ACAT1, CD36, and CPT2) were found to be up-regulated, with down-regulation of the glycolytic enzyme ENO1. This is consistent with a switch from glycolysis

to fatty acid β-oxidation as major energy source during cardiac development. However, the overall changes of the metabolic proteins were complex with some glycolytic enzymes, such as PFKM, being up-regulated following prolonged culture.[65, 66] Nevertheless, the integrated changes in metabolism are not simply reflected by down-regulation of glycolytic enzymes and up-regulation of enzymes for fatty acid oxidation. For example, one key transcriptional activator for the metabolic switch in the developing heart is the PGC-1α and PPARβ/δ complex, which both increase the expression of key enzymes in fatty acid oxidation,[65] and simultaneously increase expression of some genes related to myocardial glucose metabolism such as PFKM.[66]

At the cell surface, CD71, CD36, and TMEM65 were found to be up-regulated in hPSC-CMs after prolonged culture. CD71, also known as transferrin receptor protein 1, is responsible for iron uptake into the hPSC-CMs. Consistent with increased expression of CD71, myoglobin expression was also found increased in hPSC-CMs with prolonged culture (Fig. 5D). Myoglobin is a hemoprotein and the main oxygen carrier in the cardiac tissues and plays important roles in facilitating oxygen transport.[67] In addition to formation of myoglobin, iron homeostasis is critical for various cardiac functions, particularly electron transport and oxidative phosphorylation of the mitochondria.[68] CD36 is primarily expressed in muscles and adipocytes, and is responsible for fatty acid uptake. Recent studies also suggest a role of CD36 in cardiac recovery after myocardial infarction and Ca^{2+} regulation.[69, 70] Increased CD36 expression with hPSC-CM maturation is consistent with the switch from glycolysis to fatty acid oxidation as the main mechanism of energy production. TMEM65 is a cardiac-enriched membrane protein localized to the intercalated disc of the CMs.[71] It is essential for the formation of gap junction by facilitating correct localization of connexin 43 and thus is critical for cardiac conduction.[71] Notably, it has been shown that TMEM65 protein expression was increased during development in both mouse and human cardiac tissues, and its expression is significantly higher in ventricular than atrial tissues.[71] Collectively, TMEM65 can serve as a novel marker to assess the maturity of ventricular hPSC-CMs.

In addition to proteins involved in cellular metabolism, we found up-regulation of α-actinin 2, dystrophin, αB-crystallin, PLB and CASQ2 in hPSC-CMs following prolonged culture. These proteins are important participants in the assembly and maintenance of the contractile apparatus and sarcolemma, maintenance of mitochondrial integrity, and/or Ca^{2+} homeostasis, which are all critical for the survival and function of the mature CMs. PLB localizes to the sarcoplasmic reticulum, and plays an important role in Ca^{2+} homeostasis by regulating the SERCA pump.[72] CASQ2 also localizes to the SR and is essential for Ca^{2+} storage within this organelle. Interestingly, transgenic overexpression of CASQ2 has previously been shown to improve Ca^{2+} handling and SR maturity in hESC-CMs.[73] We collected mouse cardiac ventricular tissues at different stages of development, and confirmed up-regulation of these proteins in the developing mouse ventricles, highlighting the reliability of the proteomics method for uncovering key players involved in cardiac maturation.

The potential confounding factor for the current study is that the current differentiation protocol can generate a mixed population of CM subtypes, including nodal, atrial and

ventricular cells.[3] This could contribute to the inconsistency observed between the hiPSC-CMs and hESC-CMs. Some of these discrepancies may also result from cell line variations. However, since only one hiPSC and hESC line was analyzed, it is not possible to conclude about differences between CMs differentiated from different lines. Additionally, prolonged culturing only offered modest improvement in hPSC-CM maturity, and therefore, more advanced methods to promote hPSC-CMs are needed to further solidify the findings in this study.

In summary, we have developed an integrated proteomics method combining high-resolution top-down MS for in-depth quantification of contractile protein isoforms and PTMs, and high-throughput label-free bottom-up proteomics for in-breadth profiling of global protein expression for the assessment of hPSC-CM maturation. This method enables accurate quantification of a number of verified markers of hPSC-CM maturation which are constituents of the contractile apparatus. We applied this method to analyze hPSC-CMs from early- and late-stage monolayer and ECT cultures. Even though prolonged culturing only offered modest improvement in hPSC-CM maturity, this integrated proteomics method has identified over 200 molecular alterations during hPSC-CM maturation, including novel markers such as down-regulation of α-Tpm phosphorylation, up-regulation of cell surface markers CD36, CD71 and TMEM65, and up-regulation of proteins important for sarcomere maturation and Ca^{2+} regulation including MYOM1, αB-crystallin, α-actinin 2, PLB and CASQ2. This method is unbiased, robust, and versatile, providing a direct and comprehensive assessment of hPSC-CM maturation with high efficiency and throughput, and has laid a strong foundation for uncovering transcriptional and translational basis underlying human cardiac development, and understanding the complex disease-associated expression changes that can be modeled with patient-specific hiPSC-CMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

hPSC	human pluripotent stem cell
CM	cardiomyocyte
PTM	post-translational modification
MS	mass spectrometry
ECT	engineered cardiac tissue

hiPSC	human induced pluripotent stem cell
hESC	human embryonic stem cell
α-MHC	α-myosin heavy chain
β-MHC	β-myosin heavy chain
ssTnI	slow skeletal troponin I
cTnI	cardiac troponin I
MLC1a	myosin light chain 1 atrial isoform
MLC1v	myosin light chain 1 ventricular isoform
MLC2a	myosin light chain 2 atrial isoform
MLC2v	myosin light chain 2 ventricular isoform
cα-actin	cardiac α-actin
sα-actin	skeletal α-actin
cTnT	cardiac troponin T
LC	liquid chromatography
α-Tpm	α-tropomyosin
EIC	extracted ion chromatogram
AUC	area under curve
CV	coefficient of variation
LV	left ventricular
MLP	muscle LIM protein
CRIP2	cysteine rich protein 2
cMyBP-C	cardiac myosin binding protein C
MYOM1	myomesin 1
ENO1	enolase 1
ACAD9	acyl-CoA dehydrogenase family member 9
ACAT1	acetyl-CoA acetyltransferase
CPT2	carnitine palmitoyltransferase 2
PFKM	muscle phosphofructokinase
MLYCD	malonyl-CoA decarboxylase

MYL6	myosin light chain 6
MYH9/10	myosin heavy chain 9/10
PLB	phospholamban
CASQ2	calsequestrin 2
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase

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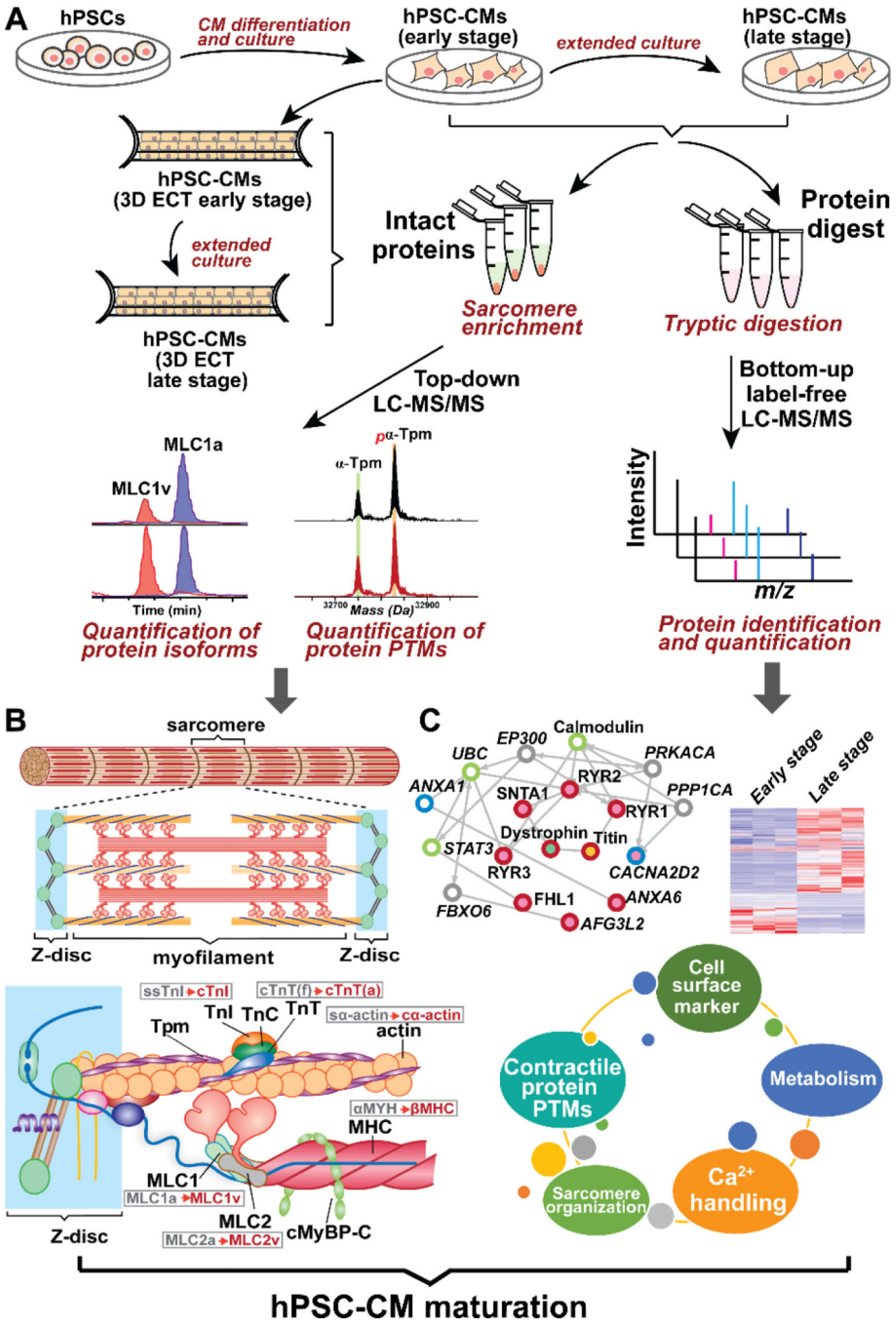

Figure 1. A comprehensive proteomics platform for the assessment of hPSC-CM maturation.

A) Experimental scheme for integrated top-down and bottom-up proteomics analyses of hPSC-CMs from early- and late-stage 2D monolayer culture and 3D engineered cardiac tissue (ECT). **B)** Top-down LC-MS-based proteomics for concurrent quantification of contractile protein isoform expression and PTMs. cTnT(f)/(a), cardiac troponin T fetal/adult isoform; sα-actin/cα-actin, skeletal/cardiac α-actin; ssTnI/cTnI, slow skeletal/cardiac troponin I; α-MHC/β-MHC, α/β myosin heavy chain; MLC1a/MLC1v, myosin light chain 1 atrial/ventricular isoform; MLC2a/MLC2v, myosin light chain 2 atrial/ventricular isoform.

Grey and red font represent fetal and adult isoforms, respectively, in the ventricular CMs. **C)** Bottom-up label-free quantitative proteomics for determining global differential regulation of proteins.

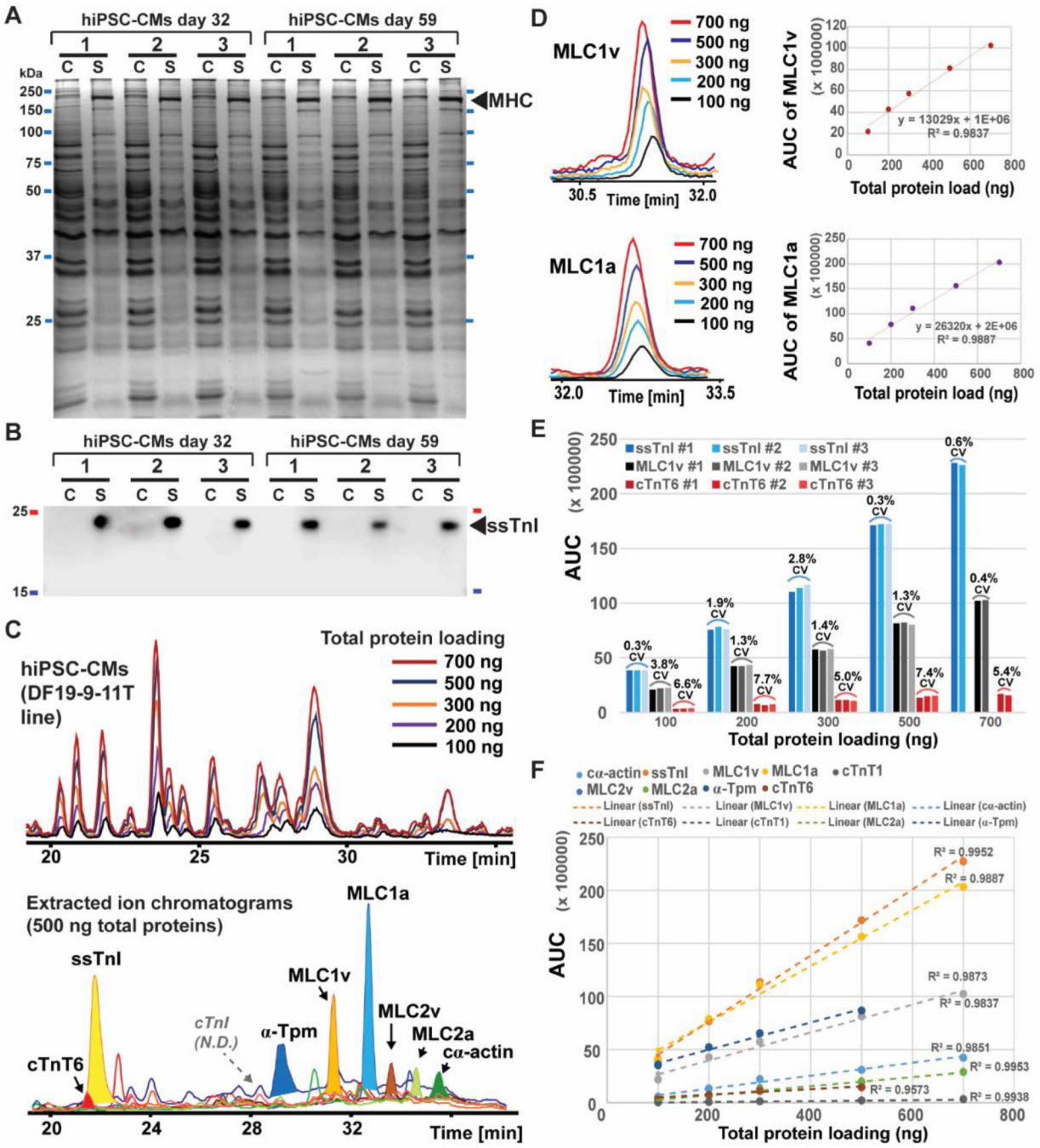

Figure 2. A robust and quantitative LC-MS-based top-down proteomics method for the quantification of sarcomeric protein isoforms from hPSC-CMs.

A) Assessment of the reproducibility of sample preparation method. 2 μg of proteins from the cytosolic and sarcomeric protein-enriched extracts were loaded and separated via SDS-PAGE, followed by silver staining. Cytosolic (C) and sarcomeric (S) protein fractions from three technical replicate extraction sof hiPSC-CMs at day 32 or 59 are highly consistent. **B)** Western blotting showing the presence of ssTnI exclusively in the sarcomeric protein fraction. **C)** Top: base peak chromatograms (BPCs) of LC-MS analysis of hiPSC-CM

sarcomeric protein fraction with various amount of total protein loading. Bottom: extracted ion chromatograms (EICs) of the representative sarcomeric proteins at 500 ng of total protein loading of the sarcomeric protein-enriched fraction. The abundance of the protein is represented by the area under curve (AUC) of the corresponding EICs. N.D., not detected. cTnT6/cTnT1, cardiac troponin T splicing isoform 6 (adult)/1 (fetal). **D)** EICs of MLC1v and MLC1a with various amount of total sarcomeric protein loading demonstrating linear response of the Q-TOF instrument. **E)** Instrument reproducibility and stability assessment with three injection replicates of varied sarcomeric protein loading. The LC-MS-based quantification of three representative sarcomeric proteins demonstrate high consistency with less than 8% coefficient of variation (CV). **F)** Linear curves of the representative sarcomeric proteins.

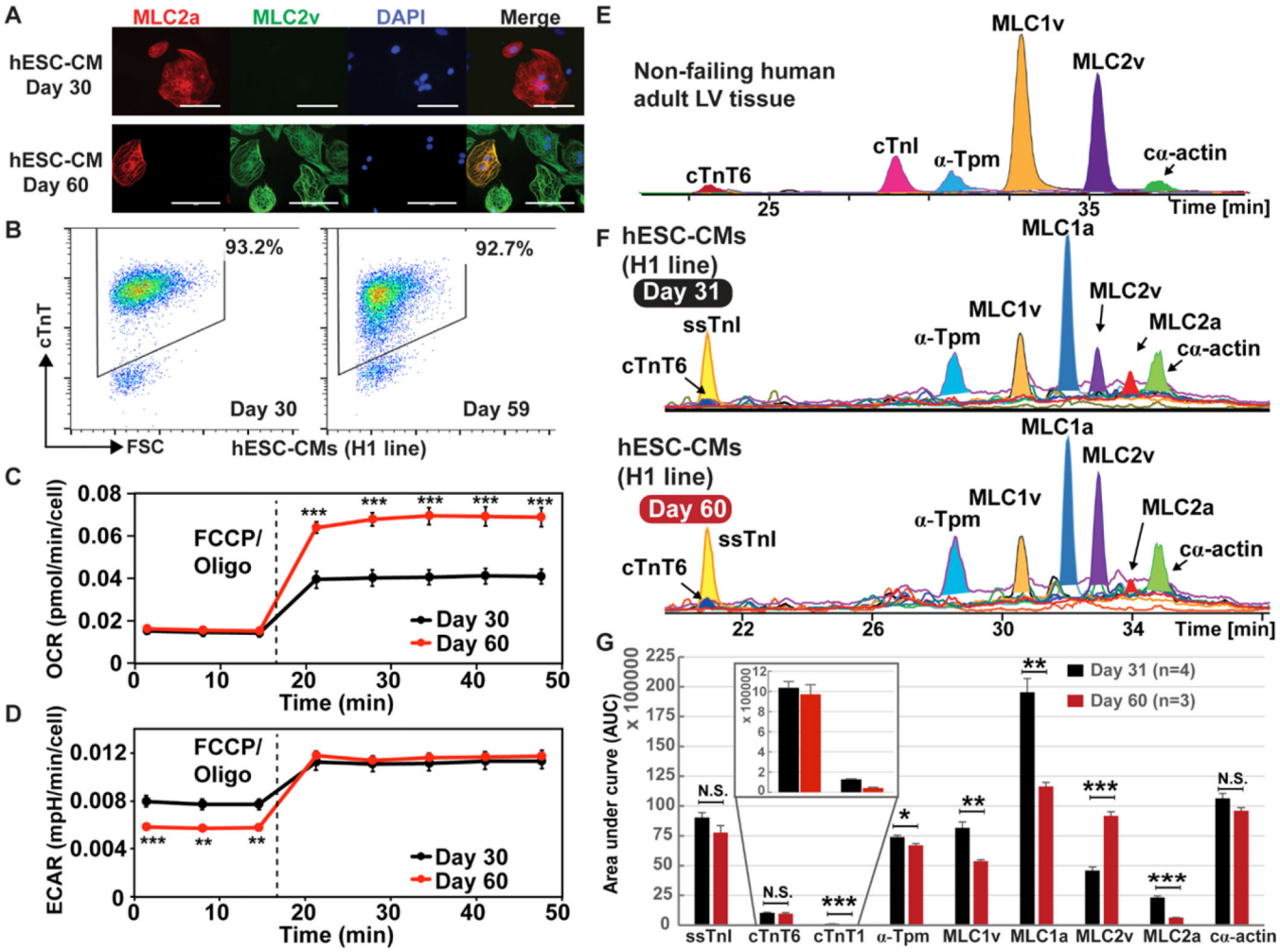

Figure 3. Assessment of sarcomeric protein isoform expression changes as markers of maturation in hESC-CMs with prolonged 2D monolayer culture.

A) Immunostaining showing increased MLC2v expression and decreased MLC2a expression in hESC-CMs (H1 line) at Day 60 compared to the cells at Day 30. Scale bar represents 100 μm. **B)** Flow cytometric analysis of hESC-CMs at Day 30 and 59 showed comparable CM purity between the early- and late-stage culture of hESC-CMs. **C)** OCR profiles of Day 30 and 60 hPSC-CMs in monolayer culture demonstrating basal OCR and maximal OCR reserve capacity. **D)** ECAR profiles of Day 30 and 60 hPSC-CMs in monolayer culture before and after addition of FCCP/Oligo. ** $p< 0.01$ and *** $p< 0.001$ by student's two sample *t*-test (two-tailed). **E)** EICs of individual sarcomeric proteins in non-failing human adult LV tissue based on top-down LC-MS analysis. **F)** EICs of individual sarcomeric proteins in hESC-CMs at Day 31 and 60 based on top-down LC-MS analysis. An equal amount (400 ng) of total protein was analyzed. N.D., not detected. **G)** Quantification of protein isoform expression in hESC-CMs at Day 31 and 60. Normal distribution of data was confirmed using Q-Q plots with Shapiro-Wilk test to compare the data with theoretical normally distributed data. * $p< 0.05$; ** $p< 0.01$ and *** $p< 0.001$ by student's two sample *t*-test (two-tailed). N.S. not statistically significant.

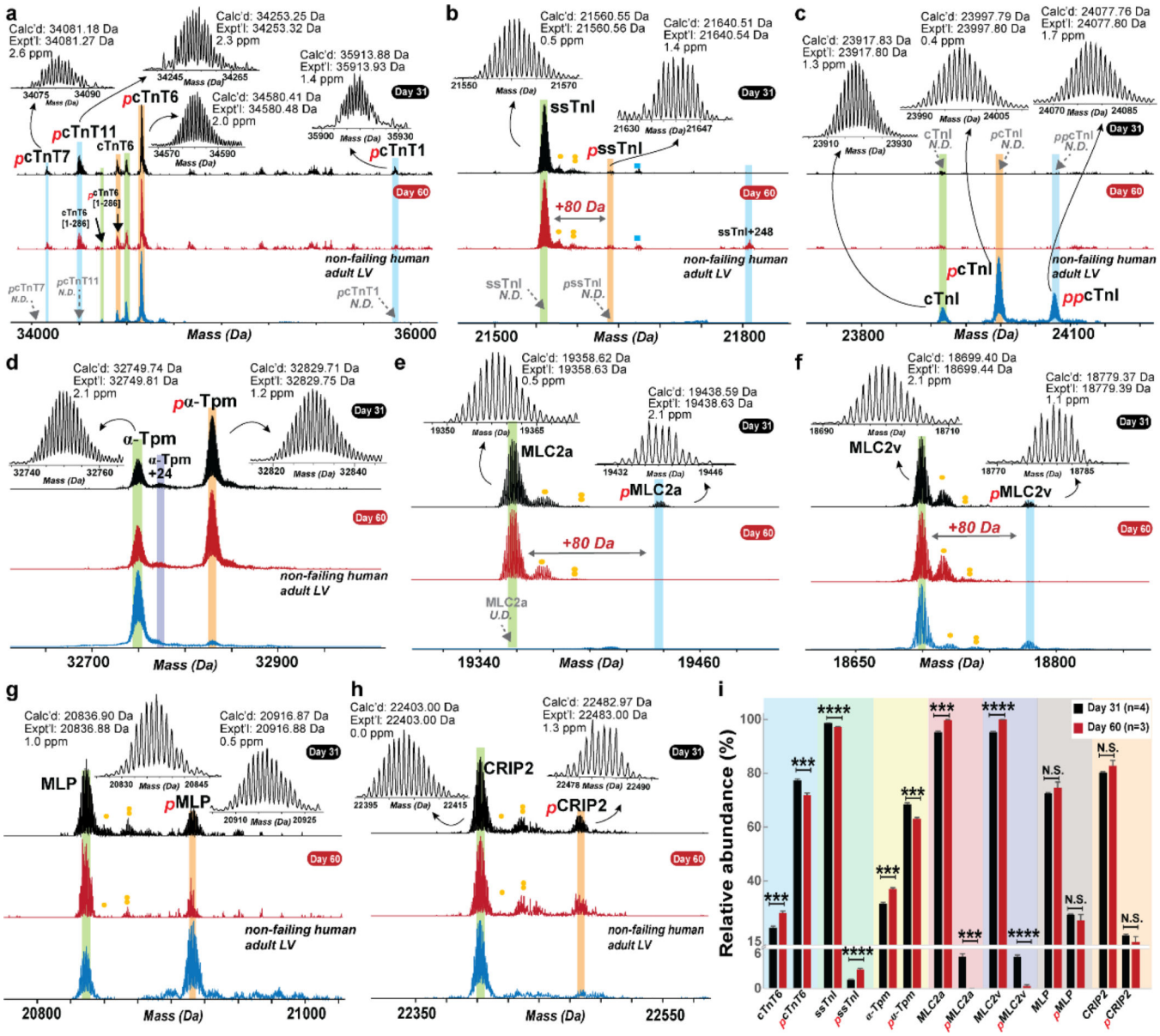

Figure 4. Relative quantification of the sarcomeric protein PTMs in hESC-CMs from 2D culture.

A-H) Comparison of the top-down mass spectra of cTnT, ssTnI, cTnI, α-Tpm, MLC2a and MLC2v, muscle LIM protein (MLP), cysteine-rich protein 2 (CRIP2), and their PTMs in hESC-CMs (H1 line) at Day 31 and 60, and non-failing human adult LV tissue. Yellow single and double circles represent singly and doubly oxidated species, respectively. Blue square represents non-covalent adductation from TFA. Italic red "p" represents phosphorylation. Cal'd, calculated most abundant mass based on protein sequence. Expt'l, experimental (observed) most abundant mass. **I)** Quantification of the relative abundance of the representative unmodified and modified sarcomeric proteins. N.D., not detected. Normal distribution of data was confirmed using Q-Q plots with Shapiro-Wilk test to compare the data with theoretical normally distributed data. *** $p < 0.001$ and **** $p < 0.0001$ by student's two sample *t*-test (two-tailed). N.S. not statistically significant.

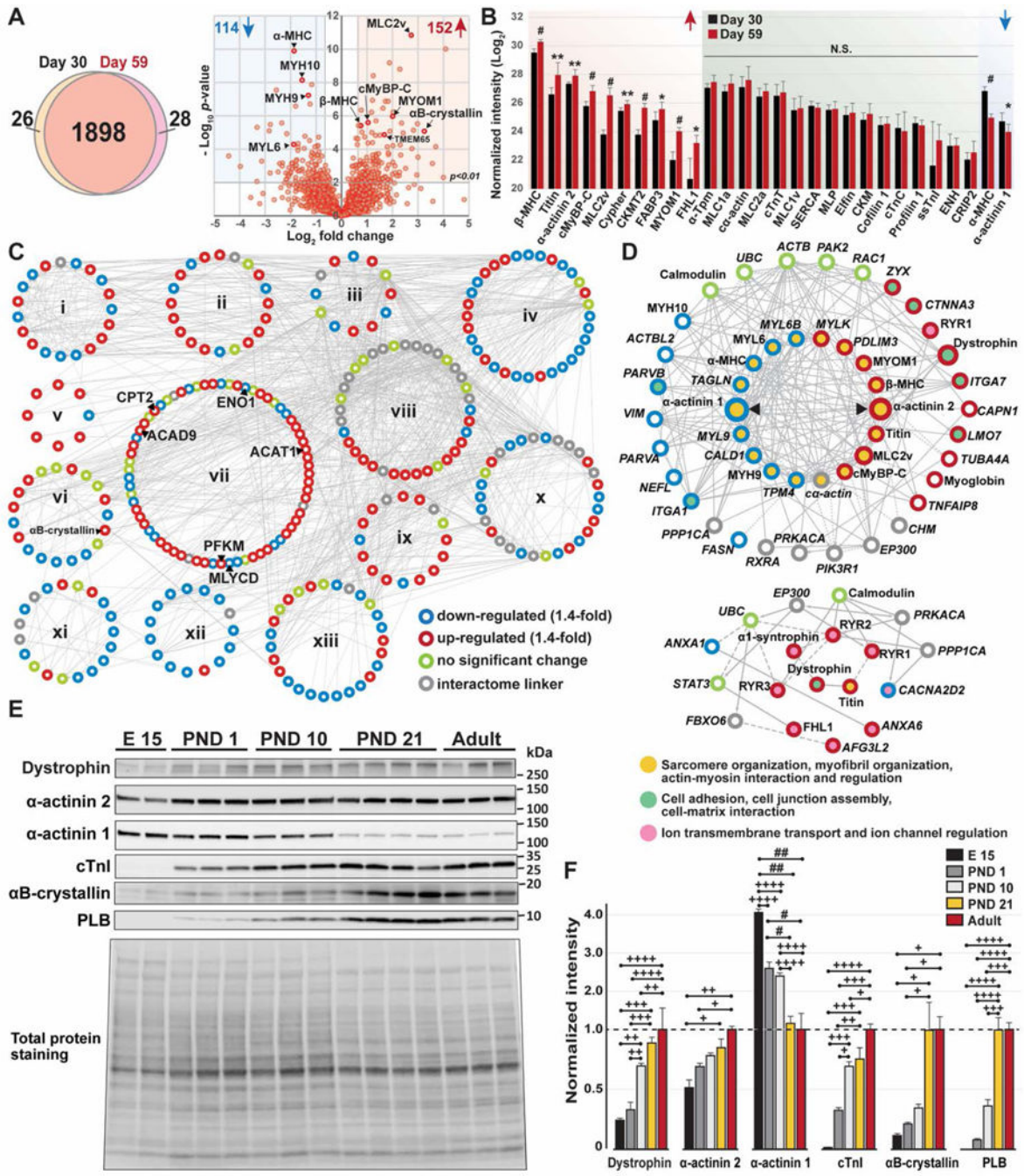

Figure 5. Large-scale bottom-up label-free quantification revealed global differential regulation of proteins in hiPSC-CMs with prolonged culture.

A) Venn diagram showing the numbers of proteins identified in hiPSC-CMs (DF19–9-11T line) at Day 30 and Day 59. Volcano plot showing differential regulation of proteins in Day 59 compared to Day 30 hiPSC-CMs. **B)** Label-free quantification of the representative contractile proteins and proteins involved in Ca^{2+} handling in hiPSC-CMs. CKMT2, sarcomeric mitochondrial creatine kinase; FABP3, fatty acid binding protein found in the heart; MYOM1, myomesin 1; FHL1, four and half LIM domain protein; SERCA,

sarcoplasmic reticulum Ca^{2+}-ATPase; CKM, creatine kinase M-type; ENH, enigma homolog. N.S, not statistically significant, * p<0.01, ** p< 0.001, *** p< 0.0001, # p< 0.00001 by the Welch's modified t-test. **C)** Functional categorization and interaction analyses of differentially regulated proteins in hiPSC-CMs in Day 59 compared to Day 30 cells. Protein grouping was based on (i), sarcomere organization, myofibril organization, or actin-myosin interaction and regulation; (ii), cell adhesion, cell junction assembly, or cell-matrix interaction; (iii), protein ubiquitination/deubiquitination, proteasome-mediated protein catabolic process, or proteasome assembly; (iv), cytoskeleton organization, or cellular component movement and organization; (v), ion transmembrane transport and ion channel regulation; (vi), protein folding, unfolded protein response, or chaperone/co-chaperone-mediated protein assembly; (vii), metabolic processes, cellular energy production, or oxidation-reduction processes; (viii), response to stimuli and signal transduction; (ix), DNA damage response, DNA replication, cell cycle regulation, or cell division; (x), regulation of transcription and gene expression; (xi), ribosome assembly and protein translation, rRNA processing, or tRNA processing; (xii), mRNA processing, transport or metabolism; (xiii), protein transport and localization, vesicle-mediated transport, or endocytosis. **D)** Extended interactome analyses of proteins involved in sarcomere organization, myofibril organization, or actin-myosin interaction and regulation, and proteins involved in ion transmembrane transport and ion channel regulation. Italic font denotes gene names. RYR, ryanodine receptor. **E)** Immunoblotting showing protein expression change in mouse ventricles during development. PLB, phospholamban. E 15, embryonic day 15; PND 1/10/21, post-natal day 1/10/21; adult, 3-month old. **F)** Quantification of protein expression during mouse ventricular development. Biological replicates for E 15, PND 1, PND 10, PND 21 and adult animals were 2, 3, 3, 4, and 3, respectively. Normal distribution of data was confirmed using Q-Q plots with Shapiro-Wilk test to compare the data with theoretical normally distributed data. + p< 0.05, ++ p< 0.01, +++ p< 0.001, ++++ p< 0.0001, # p< 0.00001, ## p< 0.000001 by one-way ANOVA with Tukey post-hoc testing of significance.

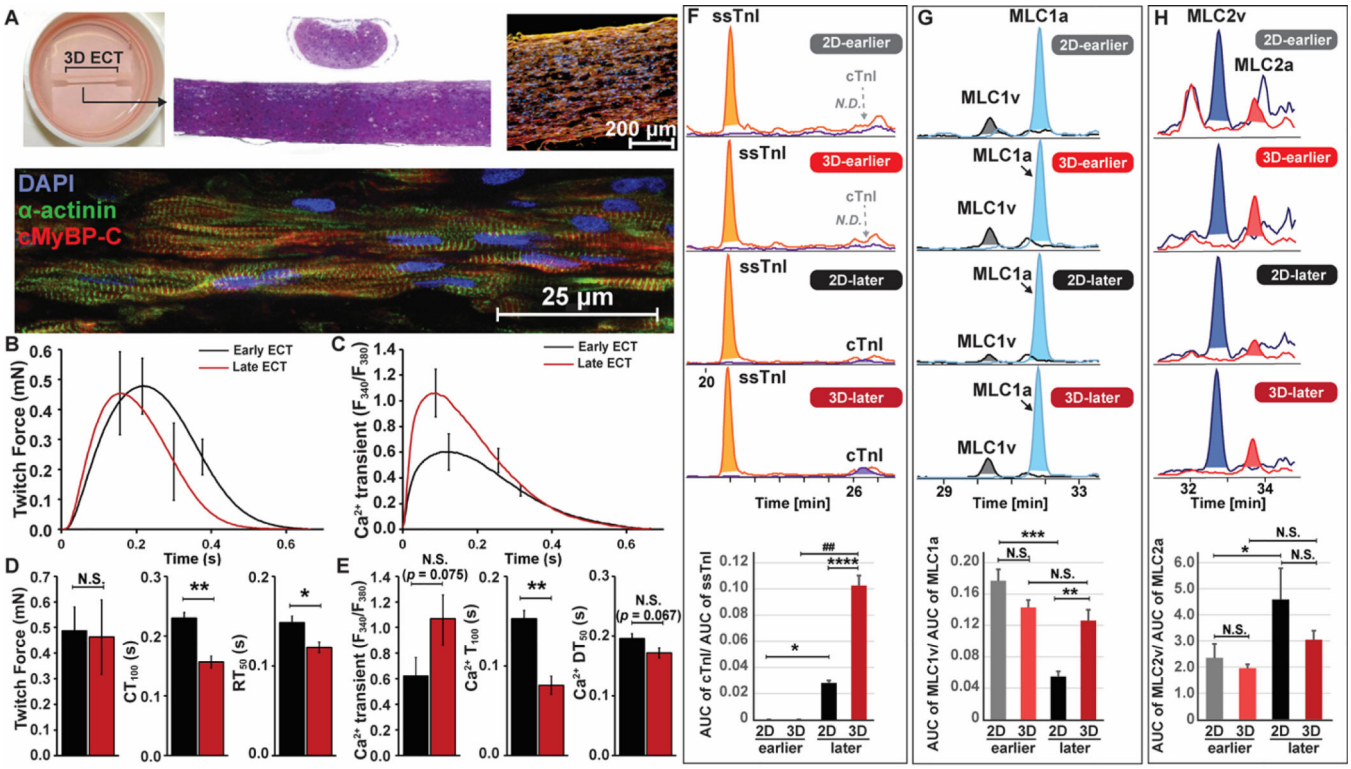

Figure 6. Quantification of sarcomeric protein isoforms in hiPSC-CMs from 2D monolayer and 3D ECT culture.

A) ECT cultured in 6-well plate and the hematoxylin-and-eosin-stained cross section and longitudinal section showing the hiPSC-CMs (DF19–9-11T line) within the ECT construct. Immunostaining showing the longitudinal orientation of the hiPSC-CMs and the sarcomere organization. Averaged twitch force **(B)** and Ca^{2+} transient traces **(C)** in early (black traces; 44–48 day old) and late (red traces; 60–78 day old) ECTs paced at 1.5Hz at 37°C. **D)** Quantification of twitch force parameters, including peak twitch force amplitude, time to peak twitch force (CT_{100}), and time to 50% twitch force decay (RT_{50}), in early and late ECTs. **E)** Quantification of Ca^{2+} transient parameters, including peak Ca^{2+} transient amplitude, time to peak cytosolic Ca^{2+} ($Ca^{2+}T_{100}$), and time from peak to 50% Ca^{2+} transient decay ($Ca^{2+}DT_{50}$), in early and late ECTs. Ca^{2+} transient amplitude is expressed as the 340/380 florescence ratio (dR). Note that ECT cultures started at Day 30 post-differentiation. Black lines and bars – early (44–48 day old) ECTs (N=13); red lined and bars – late (60–78 day old) ECTs (N=5); error bars indicate SEM; * $p< 0.05$ and ** $p< 0.01$ by Wilcoxon rank-sum test, which does not assume normality of data. **F-H)** EICs of (**F**) ssTnI and cTnI, (**G**) MLC1v and MLC1a, and (**H**) MLC2v and MLC2a from hiPSC-CMs cultured in 2D monolayer or 3D ECT at Day 44 (earlier) or Day 76 (later) post-differentiation. Noted that ECT cultures started at Day 30 post-differentiation. N.D., not detected. Normal distribution of data was confirmed using Q-Q plots with Shapiro-Wilk test to compare the data with theoretical normally distributed data. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$, # $p< 0.00001$, ## $p< 0.000001$ by two-way ANOVA. N.S. not statistically significant.