Investigating the role of the CCT chaperonin in stem cell identity and aging

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Amirabbas Khodakarami

aus Hamedan, Iran

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Berichterstatter: Dr. David Vilchez
Prof. Dr. Thorsten Hoppe

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ای دوست بیا تا غم فردا نخوریم

وین یکدم عمر را غنیمت شمریم

فردا که ازین دیر فنا درگذریم

با هفت هزار سالگان سر بسریم

خیام

O friend, for the morrow let us not worry
This moment we have now, let us not hurry
When our time comes, we shall not tarry
With seven thousand-year-olds, our burden carry.

Omar Khayyam

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Abbreviations

°C	degrees Celsius
aa	amino acid
AD	Alzheimer’s disease
BDNF	brain-derived neurotrophic factor
CCT	chaperonin containing TCP-1
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
CGC	Caenorhabditis Genetics Center
CLIPS	chaperones linked to protein synthesis
CMA	chaperone mediated autophagy
CR	caloric restriction
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DR	dietary restriction
E.coli	Escherichia coli
ER	endoplasmic reticulum
ESCs	embryonic stem cells
HD	Huntington’s disease
hESCs	human embryonic stem cells
hMSCs	human mesenchymal stem cells
HR	homologous recombination
HSCs	hematopoietic stem cells
HSF-1	heat shock transcription factor 1
HSP	heat shock protein
HSR	heat shock response
HTT	Huntingtin
ICM	inner cell mass
IGF-1	Insulin-like growth factor 1
IIS	Insulin/Insulin-like growth factor signaling
iPSCs	induced pluripotent stem cells
IR	Ionizing radation
KD	knockdown

kDa	kilo Dalton
KO	knockout
L	Liter
LB	lysogeny broth
LFQ	label free quantification
M	molar
MEF	mouse embryonic fibroblasts
Mg	Magnesium
Min	minutes
mL	milliliter
mRNA	messenger RNA
N2	wild type Bristol C. elegans strain
NAC	nascent chain associated complex
NG	normal growth
NHEJ	non-homologous end joining
NPC	neural progenitor cells
OE	overexpression
ORF	open reading frame
PBS	phosphate based saline
PCR	polymerase chain reaction
PD	Parkinson’s disease
PN	proteostasis network
Proteostasis	protein homeostasis
PSCs	pluripotent stem cells
qPCR	quantitative PCR
RAC	ribosome associated complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	reverse transcription
SCs	stem cells
shRNA	short hairpin RNA
sHSP	small heat shock protein
TRiC	TCP-1 Ring Complex

UPR	unfolded protein response
UPR^{er}	endoplasmic reticulum unfolded protein response
UPR^{mt}	mitochondrial unfolded protein response
UPS	ubiquitin/proteasome system
UV	ultraviolet
W/O	without
WT	wildtype
µg	microgram
µl	microliter
µm	micrometer

Abstract

With the constant increase in the age of the population in many countries of the world, understanding the molecular mechanisms of aging is of central importance. This increase in the age of societies in developed countries has been achieved through improvements in healthcare and drug development. However, aging is associated with a myriad of diseases that not only affect the quality of life for the elderly, but also impose a major challenge on the socio-economic and healthcare systems. Thus, gaining a better understanding of the aging process at the molecular level could lead to novel therapies against aging and age-associated diseases.

Functional decay of somatic stem cells, which leads to hampered tissue regeneration is one of the main hallmarks of aging. Human embryonic stem cells (hESCs) can replicate indefinitely while maintaining their undifferentiated state and, therefore, are immortal in culture. This capacity is among others dependent on enhanced protein homeostasis (proteostasis) mechanisms, since any imbalance in protein quality control could potentially compromise the resulting lineage of cells. Decline in the proteostasis is also known as a main contributor to aging. Thus, studying proteostasis of hESCs is a novel paradigm, that will not only help us gain a better understanding of hESC biology, but could also provide a link between hESC immortality, organismal longevity and stress resistance.

In the frame of this study, we aimed to investigate the regulation of proteostasis in hESCs and its relevance to stemness, stress resistance, aging and age-associated diseases. We tried to define key candidates responsible for enhanced proteostasis in hESCs by comparing hESCs and differentiated cells using quantitative proteomics and RNA-sequencing, and were able to identify several proteostasis-related proteins, which are differentially expressed. Recent studies demonstrate that hESCs have increased proteasome activity in comparison to their differentiated counterparts. This increased proteasome activity was found to be essential for hESC identity and their differentiation into neural cells. Elevated expression of PSMD11, a subunit of the 19S proteasome, is required for the enhanced proteasome activity in hESCs. In addition, ectopic expression of rpn-6 (*C. elegans* orthologue of PSMD11) was sufficient to confer proteotoxic stress resistance and extend lifespan in this model organism. In this study we show that PSME4, a distinct form of proteasome activator, resembles the high expression levels of PSMD11 in hESCs. However, unlike PSMD11, this protein was found to be

dispensable for stem cell function and organismal longevity under normal growth conditions.

Besides the proteasome, we also sought to understand how the chaperome regulates immortality and aging. The human chaperome is formed by 332 chaperones and co-chaperones that regulate the folding and function of proteins. We show that human pluripotent stem cells exhibit dramatic differences in expression of molecular chaperones. In particular, we observed much higher expression and increased assembly of the TRiC/CCT complex, a chaperonin that facilitates the folding of 10% of the proteome. This highly conserved molecular machine forms a~1MDa complex in the cytosol of eukaryotic cells, consisting of two back-to-back rings, each containing seven to nine subunits of ~60 kDa. TRiC/CCT is responsible for the folding of many essential proteins, including components of the cytoskeleton such as actin and tubulin or cell cycle regulators, and its dysregulation is associated with a myriad of diseases including cancer and neuropathy. We found that ectopic expression of a single subunit (CCT8) is sufficient to increase TRiC/CCT assembly. Moreover, enhanced TRiC/CCT assembly is required for the striking ability of pluripotent stem cells to maintain proteostasis of aggregation- prone huntingtin (HTT), the mutant protein underlying Huntington's disease (HD). Since the levels of CCT subunits are further decreased in somatic tissues during organismal aging, we examined whether modulation of CCT8 can delay the aging process and proteostasis dysfunction by using *C. elegans* as a model organism. Notably, upregulation of CCT8 levels in somatic tissues triggers TRiC/CCT assembly and extends organismal lifespan particularly under proteotoxic conditions. Ectopic expression of CCT8 also ameliorates the age-associated demise of proteostasis and corrects proteostatic deficiencies in worm models of Huntington's disease. Our results suggest proteostasis is a common principle that links organismal longevity with hESC immortality.

Zusammenfassung

Aufgrund der steigenden Lebenserwartungen in den Bevölkerungen vieler Länder, entwickelt sich die Erforschung der molekularen Mechanismen des Alterns zu einer wichtigen wissenschaftlichen Diziplin. Der beeindruckende Anstieg der durchschnittlichen Lebenserwartung in Industrieländern wurde durch herausragende Verbesserungen des Gesundheitswesens und der Arzneientwicklung ermöglicht. Dies ist jedoch mit extremen Herausforderungen verbunden, die das wachsende Aufkommen altersbedingter Krankheiten sowohl an das fiskale, als auch das Gesundheitssystem stellt. Ein detaillierteres Verständnis des Alterungsprozesses auf molekularer Ebene könnte daher zu verbesserten Therapien gegen solche Krankheiten im speziellen und den Alterungsprozess im Allgemeinen führen.

Funktionaler Abbau somatischer Stammzellen, welcher zu einer gestörten Geweberegeneration führt, ist eines der Kennzeichen des Alterns. Dagegen behalten menschliche embryonale Stammzellen (hES-Zellen) ihre Replikationsfähigkeit und ihren undifferenzierten Status auf unbestimmte Zeit bei und sind daher in Zellkultur theoretisch unsterblich. Diese Eigenschaft beruht unter anderem auf verstärkten Proteinhomeostase- (Proteostase-) mechanismen, da ein Ungleichgewicht in der Proteinqualitätskontrolle die resultierende Zelllinie negativ beeinträchtigen kann. Eine Abschwächung der Proteostase stellt zudem einen Hauptfaktor für den Alterungsprozess dar. Eine eingehendere Untersuchung der Proteostase von hES-Zellen dient daher nicht nur einem besseren Verständnis der hES-Zellbiologie, sondern könnte auch eine Verbindung zwischen der Unsterblichkeit von hES-Zellen, organismischer Langlebigkeit und Stressresistenz herstellen.

Das Ziel der vorliegenden Studie war die Untersuchung der Proteostaseregulation in hES-Zellen und deren Relevanz für die Stammzellenfähigkeit, die Stressresistenz, den Alterungsprozess und die Entstehung altersbedingter Erkrankungen. Durch den Vergleich quantitativer Proteomanalyse- sowie RNA-Sequenzierungdaten von hES- und differenzierten Zellen wurden mehrere potentielle Kandidatenproteine identifiziert, die in Zusammenhang mit erhöhter hES-Zellproteostase stehen.

Jüngste Studien zeigten, dass hES-Zellen eine erhöhte Proteasomaktivität im Vergleich zu differenzierten Zellen aufweisen. Diese verstärkte Proteasomaktivität ist essentiell für die Bewahrung der hES-Zellidentität und für ihre Differenzierung zu neuronalen

Zellen. Eine erhöhte Expression der 19S Proteasomuntereinheit PSMD11 ist notwendig für die verstärkte Proteasomaktivität in hES-Zellen. Darüber hinaus war die ektopische Expression von rpn-6, dem *C. elegans*-spezifische Ortholog von PSMD11, ausreichend, um Resistenz gegen proteotoxischen Stress sowie eine Verlängerung der Lebensspanne in diesem Modelorganismus zu verleihen. In dieser Arbeit zeigen wir, dass eine unterschiedliche Art von Proteasomaktivator, PSME4, ähnlich hohe Expressionslevel wie PSMD11 in hES-Zellen aufweist. Im Gegensatz zu PMSD11, ist jenes Protein jedoch verzichtbar für die Stammzellfunktion und die organismische Lebensdauer unter normalen Wachstumsbedingungen.

Das menschliche Chaperom besteht aus 332 verschiedenen Chaperonen und Co-Chaperonen, die die korrekte Faltung und Funktion von Proteinen gewährleisten. Wir zeigen, dass menschliche pluripotente Stammzellen dramatische Unterschiede in der Expression von molekularen Chaperonen aufweisen können. Wir beobachtetet im Besonderen eine deutlich verstärkte Expression sowie erhöhte Formierung des TRiC/CTT-Komplexes, einem Chaperonin, das für die Faltung von 10% des Proteoms verantwortlich ist. Diese hochkonservierte, molekulare Maschine formt einen etwa 1MDa Komplex im Cytosol eukariotischer Zellen und besteht aus zwei aufeinanderfolgenden Ringen aus jeweils sieben bis neun Untereinheiten mit einer Größe von ~60 kDA. TRiC/CCT ist für die Faltung vieler essentieller Proteine verantwortlich, darunter Komponenten des Cytoskeletts wie Actin und Tubulin, sowie Zellzyklusregulatoren. Die Dysregulierung dieses Komplexes ist mit einer Vielzahl von Krankheiten verbunden, darunter verschiedene Krebsarten und Neuropathien. Wir konnten herausfinden, dass die ektopische Expression einer einzigen Untereinheit (CCT8) ausreicht, um die Formierung von TRiC/CCT zu erhöhen. Erhöhter TRiC/CCT Aufbau war zudem notwendig für die herausragende Eigenschaft pluripotenter Stammzellen, die Proteostase des aggregationsanfälligen Huntingtin (HTT), einem Protein, dessen mutierte Form ursächlich für die Krankheit Chorea Huntington ist, aufrecht zu erhalten. Da die Zahl von CCT-Untereinheiten im somatischen Gewebe während des Alterns weiter fällt, untersuchten wir, ob die Modulation von CCT8 den Alterungsprozess und die Proteostasedysfunktion des Modelorganismus *C. elegans* hinauszögern kann. Die Hochregulierung der CCT8-Level in somatischen Geweben leitet die Formierung des TRiC/CTT-Komplexes ein und verlängert die organismische Lebensdauer besonders unter proteotoxischen Bedingungen. Ektopische CCT8-Expression verbesserte zudem den altersbedingten Abfall der Proteostase und korrigierte die proteostatischen Defizite in

Wurmmodellen von Chorea Huntington. Unsere Ergebnisse deuten auf Proteostase als gemeinsames Prinzip hin, welches organismische Lebensdauer mit der Unsterblichkeit von hES-Zellen verbindet.

1 Introduction

"It is remarkable that after a seemingly miraculous feat of morphogenesis, a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed." George Williams, Evolutionary Theorist

Understanding aging and development of remedies for it, as the ultimate cause of major diseases and death, has long been the uppermost in the minds of many. Aging research, however, has started to emerge as a scientific field only in the last three decades. This has been facilitated firstly by the discovery of specific genes, which were proven to have a direct effect on longevity, and also through the demographic shift in the developed countries. Since the second half of the 20th century, due to major improvements in medical care and drug development that leads to reduction in mortality, as well as reduced population growth rates, a steady increase in the age of the population could be observed in many developed countries. The world's population above 65 years, for instance, is expected to double in 2040 compared to 2008 (Schoeni and Ofstedal 2010). This dramatic increase in age is not only associated with many excruciating age-related pathologies such as cancer, diabetes, cardiovascular or neurodegenerative diseases for individuals, but also puts a huge burden on the socio-economic and healthcare system. It is therefore of huge importance to understand the molecular mechanisms underlying the aging process in a greater detail, so that therapies could be developed for the many age-associated pathologies, thus providing not only a longer, but also a healthier life for the elderly.

1.1 Evolution and hallmarks of aging

1.1.1 Aging from an evolutionary perspective

Aging is commonly defined as the progressive loss of physiological integrity, which leads to impaired function and increased vulnerability to environmental challenges, disease and death (López-Otín, Blasco et al. 2013) (Kirkwood 2005). Apart from species such as Hydra that do not exhibit any signs of increased mortality, reduced fertility or senescence over time (Dańko, Kozłowski et al. 2015, Schaible, Scheuerlein et al. 2015), almost all other organisms, including unicellular organisms that seem to undergo symmetrical divisions such as *Escherichia coli*, demonstrate phenotypes of aging (Stewart, Madden et al. 2005). From an evolutionary point of view, it seems counter-intuitive that such a phenomenon, which is associated with universal decline in functionality and evolutionary fitness, has not been opposed by natural selection. Theories that view aging as a programmed event, are mostly based on the argument that aging could "filter-out" the old and unfit, prevent over-crowding and thus benefit the overall fitness of the species. This theory, though supported by the observation that manipulation of a single gene could influence longevity drastically (Friedman and Johnson 1988, Kenyon, Chang et al. 1993) faces some simple but serious challenges: firstly, the huge variation in aging among individuals from single cells in a specific population, to *C. elegans* or monozygotic human twins (Kirkwood 2005) argues against a tightly programmed event. Moreover, extrinsic factors such as predation, starvation, injury or cold are considered to be the main reasons for death in natural populations (Kirkwood 2005). Additionally, individuals with mutations that turn-off the programmed aging would eventually gain evolutionary advantage over the ones with the program; and thus thrive (Kirkwood 2005). Among other theories of aging, two seem to outweigh the others: "antagonistic pleiotropy" and " the disposable soma" theory (Schulz, Palmarini et al. 2003). Antagonistic pleiotropy was put forward by the evolutionary theorist George Williams (Williams 1957) argues that genes which are advantageous in early life, could be the main drivers of aging later on. The disposable theory of aging is based on the difference in distribution of energy investment for repair and maintenance, mechanisms are highly energy consuming, in different compartments of the organism. Based on this theory, under natural conditions, more energy is allocated towards growth, reproduction and thermogenesis, and the soma needs to be maintained only as long as there is reasonable chance of survival. Minimal resources could be allocated for maintenance of somatic tissues, although this comes at the

expense of gradual damage accumulation, which will eventually lead to aging (Kirkwood 2005). The main driver of aging, time dependant stochastic accumulation of damage, is regulated and could be alleviated by energy consuming repair mechanisms, as observed in the germline or embryonic stem cells (Kirkwood 2005, Vilchez, Boyer et al. 2012, Schultz and Sinclair 2016), which are downregulated in the soma. Consistently, many lines of research show that this phenomenon is plastic and resources could be re-allocated to the soma when the organism is challenged by extrinsic factors, upon ablation of germline stem cells, for instance (Hsin and Kenyon 1999).

1.1.2 Hallmarks of aging

The orchestrated efforts to understand the molecular mechanism of aging in the recent years have shed light to many aspects of aging and potential therapies against the pathologies associated with it. This research has facilitated the definition of aging as a molecular process and its characteristics. López-Otín and colleagues summarize the hallmarks of aging as following: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (López-Otín, Blasco et al. 2013).

These hallmarks, according to López-Otín and colleagues could be categorized in three groups: primary, antagonistic and integrative hallmarks (López-Otín, Blasco et al. 2013) (Figure I). The primary hallmarks (genomic instability, mitochondrial DNA mutations, telomere loss, epigenetic alterations and loss of proteostasis) are negative and cause damage. Antagonistic hallmarks, depending on intensity could be detrimental or beneficial, which resembles the effects of hormesis (Schulz, Zarse et al. 2007, Gems and Partridge 2008, Rattan 2008, Ristow, Zarse et al. 2009, Van Raamsdonk and Hekimi 2009, De Haes, Frooninckx et al. 2014, Schaar, Dues et al. 2015, Scialò, Sriram et al. 2016). These include deregulated nutrient sensing, cellular senescence and mitochondrial dysfunction. The mitochondrial toxicity caused by higher reactive oxygen species (ROS) production, for instance, could act as a double-edged sword. In low concentrations, this ROS could act as signalling molecules, which leads to activation of pathways with overall positive outcome for the cells (Schulz, Zarse et al. 2007, Scialò, Sriram et al. 2016). However, if this limit is exceeded, the outcome would be increased cellular damage and apoptosis (Simon, Haj-Yehia et al. 2000, Circu and Aw 2010). Finally, the accumulated damage will impinge upon tissue homeostasis by

diminishing the stem cell function or dysregulated cellular communication, which shifts the balance towards development of aging (Figure I.)

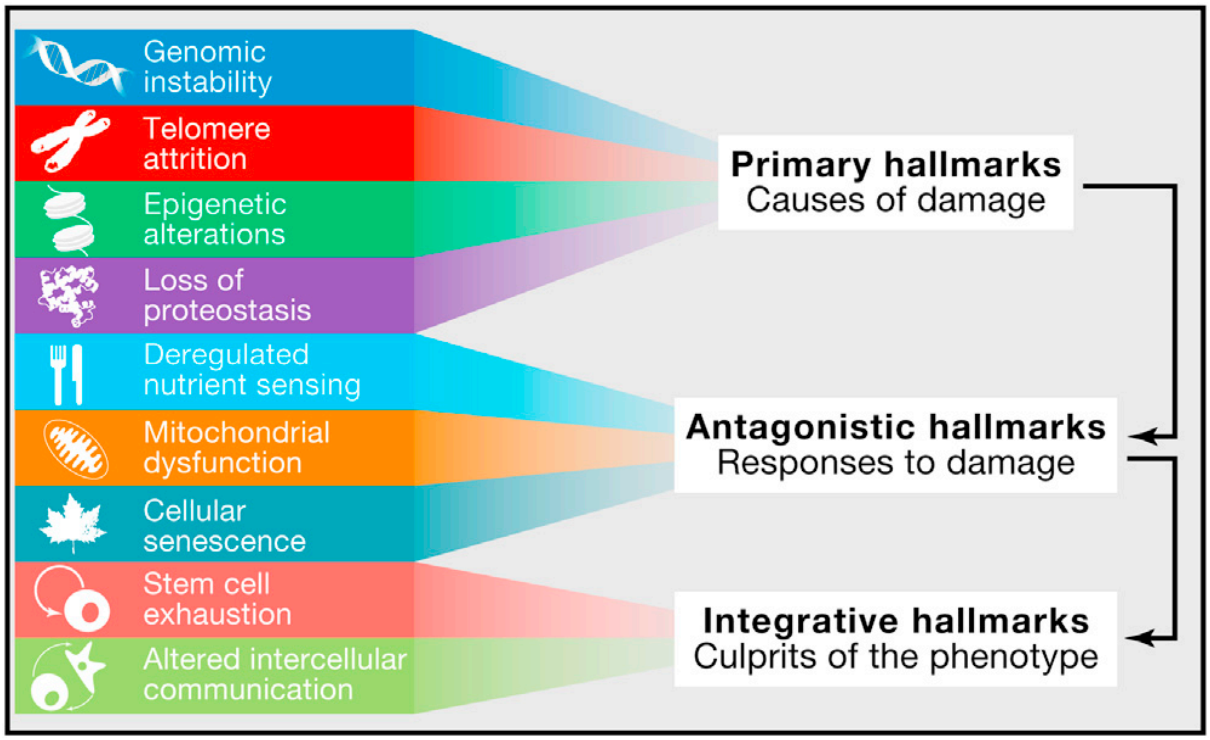

Figure I. The hallmarks of aging and their interconnection. The primary hallmarks are considered to be the main source of damage accumulation. Depending on the intensity, some beneficial stress response mechanisms could become detrimental (antagonistic hallmarks) Integrative hallmarks are the outcome of accumulated damage and chronic stress response, which eventually lead to imbalances in function and physiology of the organism and result in aging. Figure reprinted from (López-Otín, Blasco et al. 2013), with permission from Elsevier.

1.2 Proteostasis in health, disease and aging

Maintaining protein homeostasis (proteostasis) is a complex and tightly controlled task, which keeps the integrity of the proteome. Proteostasis has been one of the first biological processes which arose early after development of life on earth, and has been so central in development and evolution of life that no organism lacking mechanisms for proteostasis maintenance has survived to the present day (Powers and Balch 2013). Although unique for each species or even cell type, a proteostasis network (PN), consisted of complex and interconnected cellular mechanisms is developed to constitutively monitor the synthesis,

folding, degradation, localization and interaction of proteins (Vilchez, Saez et al. 2014). The PN, comprising approximately 1400 components in a mammalian cell (Balchin, Hayer-Hartl et al. 2016), is robustly organised and disruptions in the PN is strongly associated with diseases (Balchin, Hayer-Hartl et al. 2016). Moreover, many components of the PN decline during aging, and collapse of the proteostasis has been described as a primary hallmark of aging (Taylor and Dillin 2011, López-Otín, Blasco et al. 2013).

1.2.1 Protein quality control by ribosomes and molecular chaperones

In order to perform their normal function, most proteins need to be folded in their native three-dimensional form. Although small proteins of ~100 aa refold spontaneously in vitro, larger proteins require further assistance in order to find their correct folding (Balchin, Hayer-Hartl et al. 2016). The folding process is an error-prone process, due to the large number of possibilities how a polypeptide chain could fold (Balchin, Hayer-Hartl et al. 2016). Although folding is opposed by entropy, there are many forces including hydrophobic forces, hydrogen bonding and intramolecular interaction which favour folding into the native state (Powers and Balch 2013). It should be noted, however, that proteins often need to be assisted in overcoming kinetic barriers, until they reach this thermodynamically favourable native state (Balchin, Hayer-Hartl et al. 2016).

The native structure of proteins is constantly challenged, even by minor fluctuations in the environment such as pH or ionic strength. Moreover, a cell is not only confronted with the synthesis and folding of normal proteins, but also refolding, degradation or sequestration of misfolded, mutant or aberrant polypeptides. This challenge is even greater under stress conditions, pathology or aging (Chen, Retzlaff et al. 2011). Misfolded proteins can gain new functions which could be deleterious to the cell, or form toxic protein aggregates which will interact with many cellular compartments, overwhelm the proteostasis machinery and lead to pathology (Chen, Retzlaff et al. 2011). Aggregation of proteins is mainly caused by exposure of hydrophobic residues of a misfolded polypeptide backbone to the solvent in high concentration (Balchin, Hayer-Hartl et al. 2016). Conformational diseases, which include but are not limited to age-associated neurodegenerative disorders such as Alzheimer' (AD), Huntington' (HD) or Parkinson' (PD) diseases are caused by accumulation of misfolded proteins or metastable protein species such as amyloid fibrils (Chen, Retzlaff et al. 2011).

Thus, it is crucial for the cell to actively control and enhance the folding of proteins in order to survive.

Poteostasis begins with the folding of proteins or preventing their misfolding, simultaneously with the biosynthesis of polypeptide chains (Wolff, Weissman et al. 2014). Different mechanisms are employed at the ribosome to increase translation fidelity. Modification of the translation rate, by using alternative codons for instance, could provide more time for chaperone binding, or inversely, prevent aggregation formation by decreasing the probability of intermediate folding (Wolff, Weissman et al. 2014). Additionally, proofreading mechanisms in the tRNA and co-translational folding by ribosome binding chaperones provide further possibilities for reaching the native state (Guo and Schimmel 2013, Gloge, Becker et al. 2014).

Molecular chaperones and folding factors that influence their ATPase activity and determine the folding pathway (Chen, Retzlaff et al. 2011) are the central hub of protein folding. Chaperones constantly detect abnormalities in protein conformation or function (Powers and Balch 2013, Brandvold and Morimoto 2015). They also assist proteins in their trafficking, refold denatured proteins or target irreparable misfolded proteins for degradation (Chen, Retzlaff et al. 2011, Labbadia and Morimoto 2015). Many of the chaperones are heat shock proteins (HSPs), which are highly expressed upon exposure conformational stresses. Based on their molecular weight, HSPs are divided in six groups: sHSP, HSP40, HSP60 (chaperonins), HSP70, HSP90, and HSP100 (Brandvold and Morimoto 2015). However, not all the chaperones are induced by stress, and based on their functional clustering it has been suggested that they could be categorized in another distinct group: chaperones linked to protein synthesis (CLIPS) (Albanèse, Yam et al. 2006). The first chaperones to interact with the nascent polypeptide chain are ribosome-binding chaperones, which form two complexes: ribosome associated complex (RAC) and nascent chain associated complex (NAC) (Figure II). (Balchin, Hayer-Hartl et al. 2016). RAC and NAC bind the ribosome and the short nascent chains of the polypeptide, and couple co-translational folding with peptide elongation (Balchin, Hayer-Hartl et al. 2016). If the protein is unable to fold at the ribosome, the folding process is then continued with the action of chaperones with no interaction with the ribosome, such as Hsp70/Hsp40 system. Multiple Hsp70 chaperones or the hexameric prefoldin could act as intermediate so that the polypeptide is transferred to Hsp90 or chaperonin systems without formation of aggregation prone intermediates (Figure II). The folding process is then

finalized either by the Hsp70, the chaperonin or the Hsp90 systems (Figure II). (Balchin, Hayer-Hartl et al. 2016). The two most abundant chaperone families, Hsp70 and Hsp90 are ATP dependant chaperones, could make up to 2% of the protein content of some cells, and have organelle specific homologs which are essential for the function of the endoplasmic reticulum (ER) (BiP and GRP94) or mitochondria (Mortalin and TRAP1) (Labbadia and Morimoto 2015). Co-chaperones (such as members of the DNAJ family) enhance the folding process by either stimulating the ATPase activity of the chaperone, or by delivering the misfolded proteins to the chaperones (Labbadia and Morimoto 2015). Another family of chaperones are the small heat shock proteins (sHSPs), which play an essential role in prevention of aggregation formation by forming homo-oligomeric structures which inhibits formation of molecular interactions which could lead to aggregation formation in the cytosol (Labbadia and Morimoto 2015).

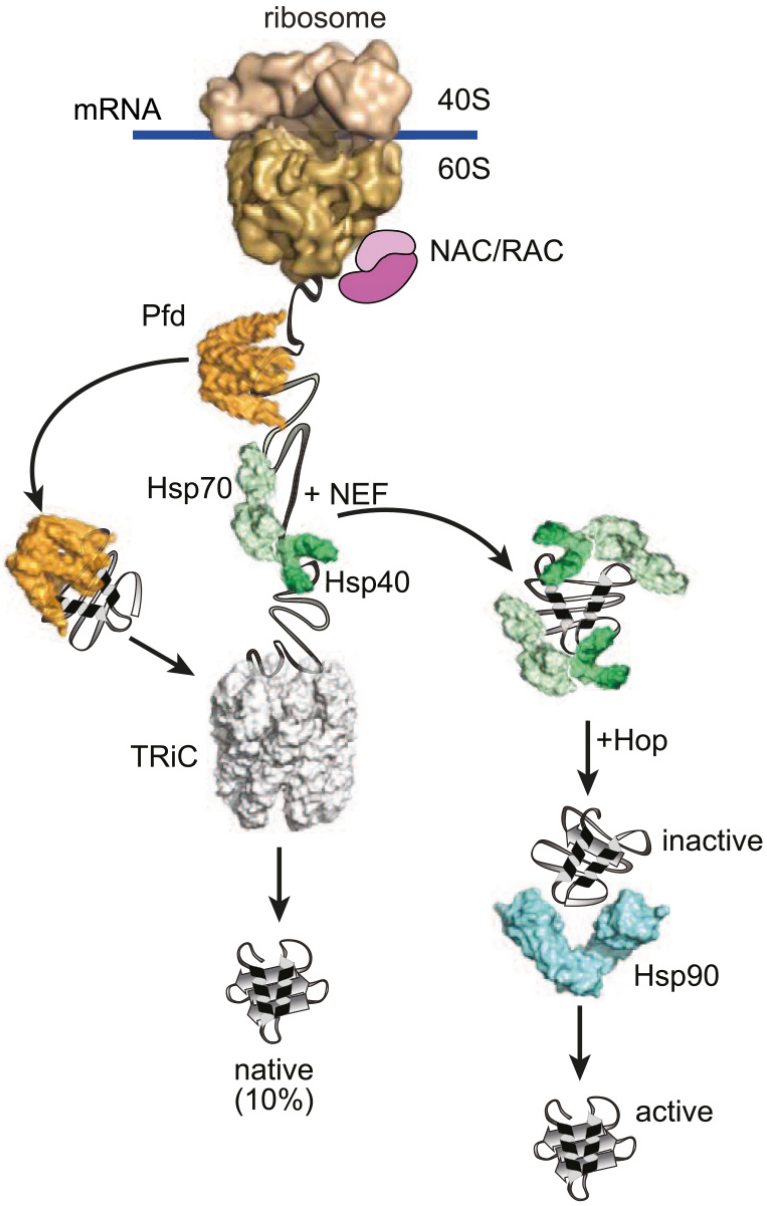

Figure II. The mechanism of protein folding in Eukaryotic cells. The majority of polypeptides (~70%) are folded upon translation. The proteins unable to fold by ribosome associated chaperones are either folded by the orchestrated action of Hsp70/Hsp90 chaperones (~20%) or the chaperonin (~10%). Image has been modified from (Balchin, Hayer-Hartl et al. 2016), with permission from AAAS.

1.2.1.1 CCT/ TRiC is a central protein folding and disaggregation machine

Almost 10% of the proteome could not be folded by the Hsp70/Hsp40 or Hsp90 systems, and require the specialized function of the Hsp60/chaperonin family to reach their native state (Labbadia and Morimoto 2015). Chaperonins are highly conserved and are essential in all domains of life (Balchin, Hayer-Hartl et al. 2016). All chaperonins form multimeric cylindrical complexes to provide a protected cavity, in which a single molecule of non-native protein can be folded (Labbadia and Morimoto 2015, Balchin, Hayer-Hartl et al. 2016). This family of molecular chaperones can be divided in two distinct groups. Group I chaperonins are present in bacteria (GroEL) and eukaryotic organelles derived from endosymbiosis: mitochondria (Hsp60) and chloroplasts (Cpn60) (Lopez, Dalton et al. 2015). Group II chaperonin, also known as class II TCP-1 Ring Complex (TRiC) or cytosolic chaperonin-containing t-complex polypeptide 1 (hereafter CCT), is found in the cytosol of eukaryotic cells. Both classes of chaperonins share similar structural characteristics forming a ~1MDa complex consisting of two back-to-back rings, each containing seven to nine subunits of ~60 kDa (Labbadia and Morimoto 2015). CCT is responsible for the folding of many essential proteins, including components of the cytoskeleton such as actin and tubulin or cell cycle regulators (Lopez, Dalton et al. 2015). All CCT subunits share a common domain structure: an equatorial domain contains inter- intra-ring contacts as well as the ATP binding site, an apical domain which harbours the substrate binding domain and the intermediate domain which serves as a link between the two other domains (Yébenes, Mesa et al. 2011). Subunit arrangement in the complex is defined by their ATP binding affinity and charge (Reissmann, Joachimiak et al. 2012). Each subunit of the CCT complex has a different affinity for ATP, and alternating distribution of subunits with high and low affinity for ATP leads to sequential action of the complex (Balchin, Hayer-Hartl et al. 2016). CCT cycles between open and closed states (Figure III). Upon binding of ATP, the complex forms the closed conformation, and the state of bound nucleotide is communicated across all the subunits of the complex. Each subunit has helical protrusions that together form an iris-like lid (Balchin, Hayer-Hartl et al. 2016). Even in the closed state, the folding cavity is not perfectly sealed as the lid provides a small pore that can accommodate polypeptide chains facilitating the binding of large substrates in a domain-wise or co-translational fashion (Balchin, Hayer-Hartl et al. 2016). Upon ATP hydrolysis, which is sensed by a conserved lysine residue, the lid of the complex forms a β-barrel to cover the folding chamber (Lopez, Dalton et al. 2015). Once ADP

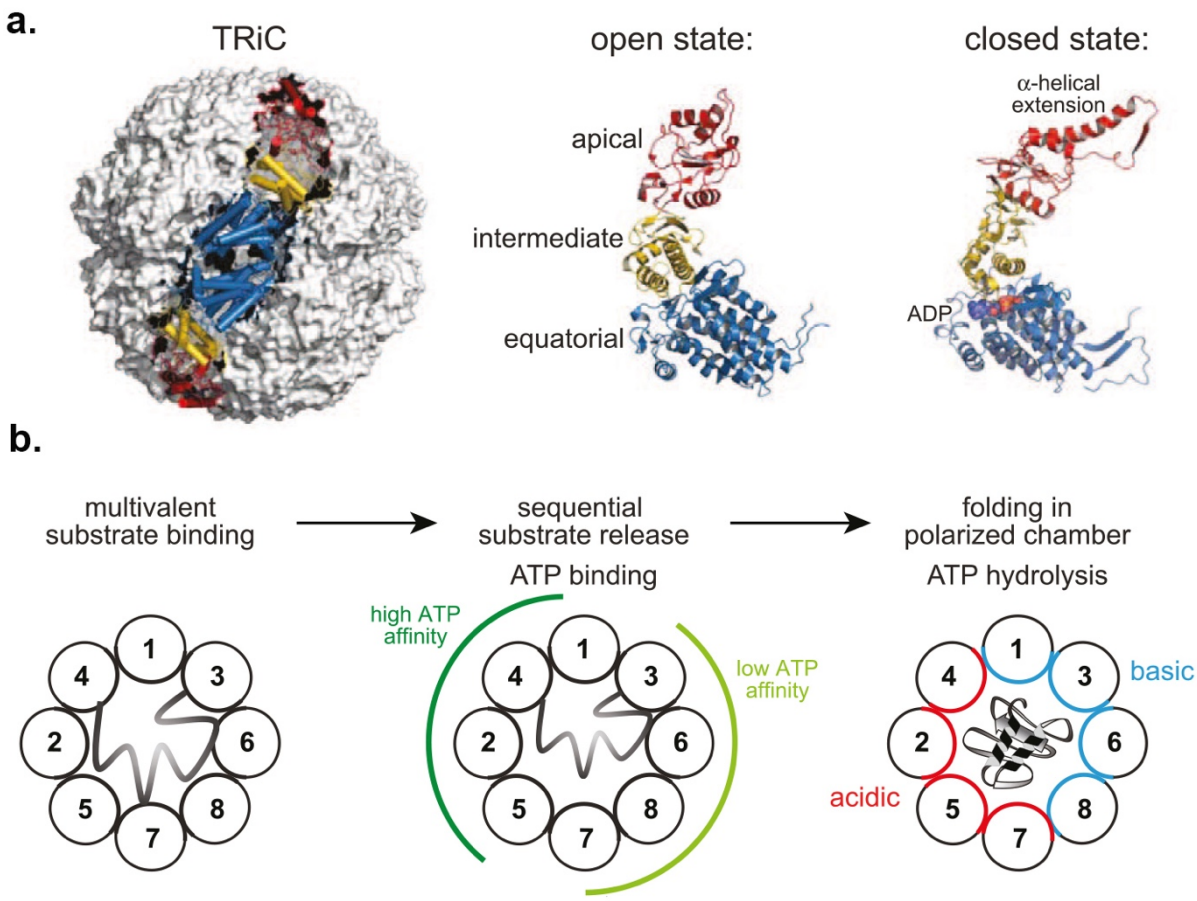

Figure III. Structural conformation and molecular mechanism of CCT/TRiC function. (a). Structure of the eukaryotic chaperonin TRiC (**b**). Mechanism of TRiC function. The subunit orientation of the TRiC complex is shown schematically as a top view of the ring. Nonnative substrate protein binds in a distinct topology contacting the apical domains of specific subunits (left). Upon ATP binding, TRiC releases the protein in a sequential manner aided by asymmetry in ATP affinities of the TRiC subunits (middle). After release, the protein folds to completion inside the TRiC cage, the hemispheres of which are partitioned into acidic and basic character. Image has been modified from (Balchin, Hayer-Hartl et al. 2016), with permission from AAAS.

is released from the active site, the lid goes into the open conformation again and the cycle continues. The hetero-oligomeric nature of this complex generates the functional asymmetry which is crucial for the folding of some specific substrates, which are unable to fold by any other chaperone system (Lopez, Dalton et al. 2015). Taken the large number of important subunits, it is clear that dysregulation of CCT is associated with a myriad of diseases including cancer and neuropathy. CCT is responsible for folding of proteins with complex topology (Yam, Xia et al. 2008) and ameliorates the cytotoxicity by changing the aggregation state in models of HD and AD (Tam, Geller et al. 2006, Khabirova, Moloney et al. 2014).

1.2.2 Protein clearance mechanisms are essential for cell survival and are implicated in aging

1.2.2.1 The proteasome is the primary protein degradation machine in the cell

In order to maintain protein homeostasis, it is absolutely essential that damaged or old proteins be degraded, so that the risk of aggregation is diminished and the amino acids could be recycled for new rounds of biosynthesis. The two main cellular proteolytic systems are the ubiquitin proteasome system (UPS) and autophagy. The UPS is the primary selective mechanism of protein degradation in eukaryotic cells (Schmidt and Finley 2014). The UPS is a carefully timed and precise mechanism which is critical for maintaining the appropriate levels of many regulatory proteins involved in several pathways such as signal transduction, metabolism or cell cycle (Finley 2009, Wong and Cuervo 2010, Buckley, Aranda-Orgilles et al. 2012, Okita and Nakayama 2012, Vilchez, Morantte et al. 2012, Tanaka and Matsuda 2014). The UPS is not only necessary to degrade regulatory proteins but it is also an essential component of the proteostasis network necessary for eliminating damaged, misfolded and aggregation-prone proteins (Finley 2009, Wong and Cuervo 2010, Tanaka and Matsuda 2014). The first step of the UPS-mediated proteolysis is the covalent conjugation of a highly conserved small protein, ubiquitin, through a sequential mechanism that targets proteins for degradation. The polyubiquitylated proteins are then recognized, unfolded and finally cleaved into small peptides by the proteasome (Vilchez, Saez et al. 2014) (Figure IV). The proteasome is a complex proteolytic machine of 2.5 MDa formed by the assembly of several subunits (Coux, Tanaka et al. 1996). The core particle (20S) of the proteasome consists of 28 subunits, which are assembled into four seven-membered rings and exhibit a barrel-like structure (Coux, Tanaka et al. 1996) (Figure IV). The two outer rings are composed by seven a-subunits (named a1-a7), while the two inner rings are composed by seven b-subunits (b1-b7). b-rings contain the proteolytic active sites: b1, b2 and b5 present caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Vilchez, Saez et al. 2014). Although 20S particles can exist in a free form, they are considered to be inactive due to its closed form and binding to proteasome activators is required for degradation of polyubiquitylated proteins (Kisselev and Goldberg 2005). However, free 20S particles can degrade small proteins in an ATP- and ubiquitination-independent manner (Baugh, Viktorova et al. 2009). The most common active proteasome results from the assembly of the 20S and the 19S (26S, single capped or 30S, double capped) (Finley 2009). The 19S regulates the activity of the complex and is responsible for recognizing, unfolding and translocating the polyubiquitylated proteins

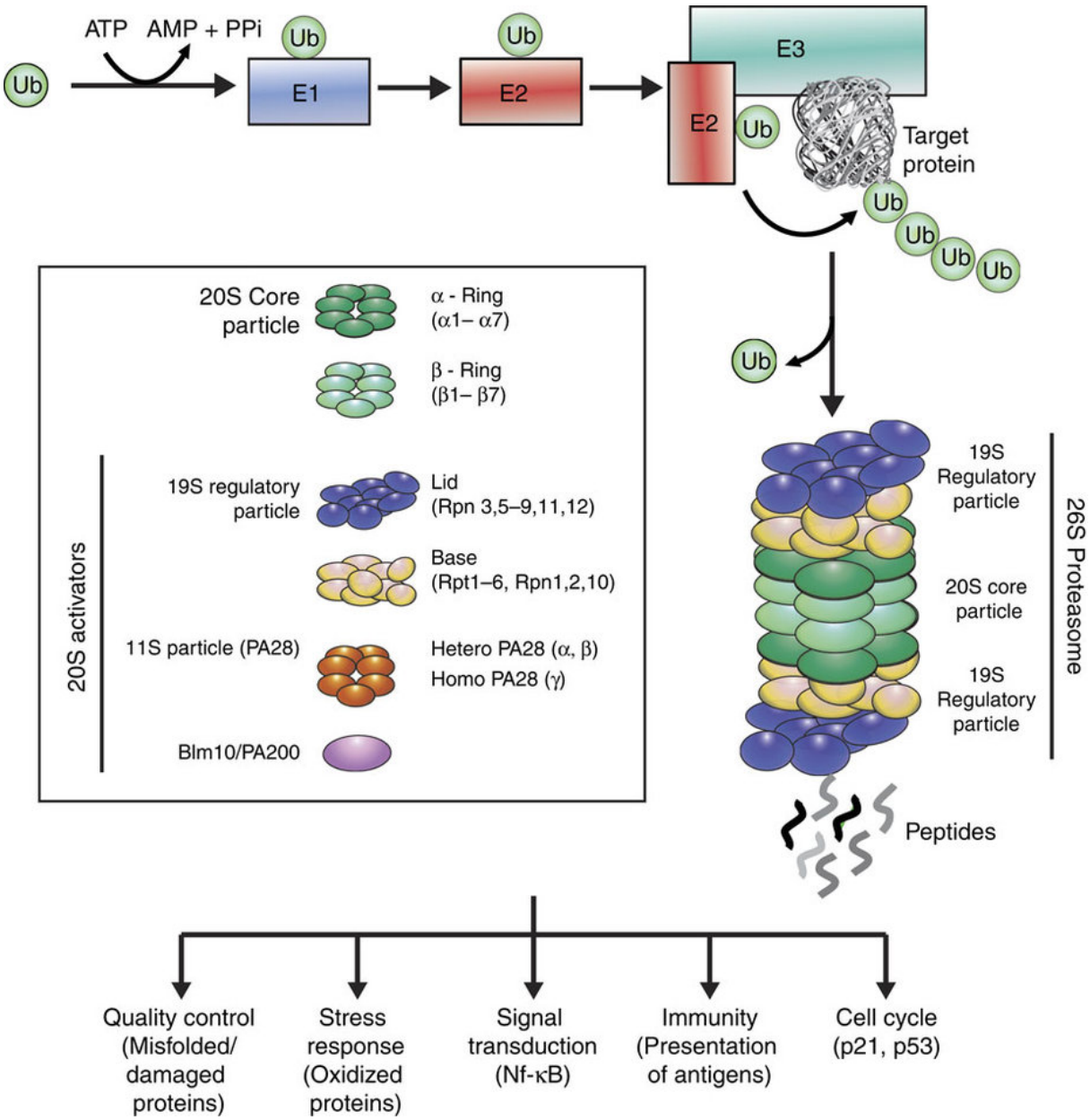

Figure IV. The UPS is one of the main protein degradation systems in the cell. Proteins targeted for proteasomal degradation are tagged as the result of orchestrated action of E1, E2 and E3 enzymes. The ubiquitinated protein is either transferred to the proteasome by external factor, or interacts directly with ubiquitin receptors of the proteasome. The protein is then unfolded, ubiquitin moieties are removed by deubiquitinating enzymes, and pulled into the catalytic chamber to be cleaved into short peptides. Depending on the 20S activator, the UPS is also capable of degrading unubiquitinated or unstructured polypeptide chains. The wide range of UPS substrates are implicated in almost all important cellular processes. Image reprinted from (Vilchez, Saez et al. 2014), with permission from Nature Publishing Group.

to the 20S for degradation.in an ATP dependent manner (Finley 2009, Tanaka and Matsuda 2014). Notably, proteins and even protein aggregates can also be degraded in an ubiquitin-independent way by free 20S or by 20S particles activated by other regulatory particles such

as PA28 or PSME4 (also known as the proteasome activator 200, PA200) (Dubiel, Pratt et al. 1992, Ma, Slaughter et al. 1992, García-Mata, Bebök et al. 1999, Baugh, Viktorova et al. 2009). PSME4 is a monomeric protein of 250 kDa and in contrast to the 19S regulatory particle; can activate the core proteasome without having ATPase or ubiquitin binding properties. PSME4 can form hybrid complexes in which this protein binds to one end of the 20S proteasome and the 19S to the opposite end. PSME4 has been suggested to be essential for spermatogenesis and DNA repair, but the precise biological functions remain controversial and poorly understood.

1.2.2.2 Autophagy: the bulk degradation pathway

Parallel to the proteasome, autophagy is the main cellular protein clearance pathway. Although the UPS is considered to be the main protein degradation system in the cell, it is unable to degrade large protein complexes or aggregates (Labbadia and Morimoto 2015). Cytosolic fractions, organelles and macromolecules can be degraded by autophagy through the lysosome. Depending on cargo recognition system and the compartment it is delivered to, autophagy could be sorted in different categories. Macroautophagy is defined as engulfment of organelles or cytosolic regions by a double membrane structure known as autophagosome, which then fuses with the lysosome leading to degradation of its contents (Wong and Cuervo 2010, Labbadia and Morimoto 2015). Microautophagy is defined as parts of the cytosol is directly sequestered by the lysosome, and chaperone mediated autophagy (CMA) which occurs through direct delivery of target proteins by a chaperone such as Hsc70 to the lysosome. These distinct forms of autophagy were initially believed to be induced upon stress, but recent evidence suggest a constitutive basal autophagic activity in most cell types (Wong and Cuervo 2010). Also in energy-demanding situations, such as nutrient-deprivation, autophagy degrades many different substrates to fulfil the energetic requirements of the cell (Egan, Kim et al. 2011, Rubinsztein, Mariño et al. 2011). These substrates include macromolecules that provide energy and nutrients during starving periods (Ravikumar, Sarkar et al. 2010). Moreover, due to its capacity to engulf whole cellular regions, autophagy is essential in processes that require extensive cellular restructuration, such as embryogenesis, cellular differentiation or cellular death (Cuervo 2004, Mizushima, Levine et al. 2008). The catalytic components of autophagy are the lysosomes. Lysosomes contain a large variety of cellular hydrolases, proteases, lipases, nucleotidases and glycosidases that show highest activity at acidic pH. Autophagy is required for maintaining cellular homeostasis, acting as a quality control mechanism of proteins and organelles. Moreover, lysosomal proteolysis results

in small di- and tripeptides and free amino acids that are released into the cytosol to be further metabolized to obtain energy or recycled to synthesize the novo proteins (Ravikumar, Sarkar et al. 2010, Mizushima and Komatsu 2011).

1.2.2.3 Stress response pathways are a safeguard against proteotoxicity

The PN is a very dynamic network and has been evolved to be able to protect the cell against proteotoxic stresses that cause protein misfolding, such as temperature shift or inflammation. Under such conditions, cells exhibit the capability of rapidly altering their PN by increasing the levels of molecular chaperones, co-chaperones and proteasomal subunits, to meet the transient necessity of responding to the proteostatic burden (Labbadia and Morimoto 2015). For instance, the expression of HSP70 increases up to 20,000 fold at the transcript level in most cases of heat shock (Powers and Balch 2013) This rapid, stress-induced alterations in the PN include a global heat shock response (HSR) and unfolded protein responses (UPR) in the ER (UPR^{ER}) and mitochondria (UPR^{mt}) of eukaryotic cells (Powers and Balch 2013).

The HSR relies mainly on the action of sensitive heat shock transcription factors (HSF), that are maintained in an inactive state, for instance in a complex with a chaperone such as HSP90 or HSP70 (Powers and Balch 2013, Labbadia and Morimoto 2015). HSF1, which is considered to be the master regulator of HSR, for instance, is released from the chaperone upon protein denaturing stress, acquires DNA binding ability through homotrimerization and translocates to the nucleus in order to induce the transcription of many genes encoding molecular chaperones. When the stress is diminished, HSF1 activity is dampened by its acetylation, which again results in its binding to molecular chaperones (Labbadia and Morimoto 2015). Although distinct HSFs and pathways are involved, the same concept is relatively valid for HFSs responsible for activation of UPR^{ER} and UPR^{mt}. These stress response pathways are essential for normal cellular physiology, as supported by the fact that HSF1 is essential for development in all domains of life (Labbadia and Morimoto 2015). Indeed, the importance of the proteome integrity is so high for multicellular organisms that proteotoxic stress is constantly monitored and signaled through the whole organism by complex mechanisms to ensure the optimal proteostasis both within (cell-autonomous) or between cells and tissues (cell-non autonomous) (Labbadia and Morimoto 2015). Downregulation of a mitochondrial complex IV subunit in neurons, for instance, can activate the UPR^{mt} in the intestine of *C. elegans*, and enhance organismal longevity (Taylor,

Berendzen et al. 2014). Similar cell-non-autonomous mechanisms exist for HSR and UPR^{ER}, and the molecular nature of this phenomenon is beginning to be understood. Although the transient stress response increases the overall fitness of the cell or organism, tight regulation of these pathways is crucial for preventing the detrimental consequences of constitutive HSR, which is linked to multiple pathologies including many types of cancer (Labbadia and Morimoto 2015).

1.2.2.4 Impaired proteostasis causes disease and contributes to aging

Many human disorders can be categorized as PN disruptions. Amyloid fibrils are insoluble protein aggregates that are formed in the nucleus and cytoplasm of neurons. These inclusions are composed of large ordered fibrils of metastable β-sheet-enriched proteins that are believed to have a causative role in around 50 human diseases including HD, PD, AD (Labbadia and Morimoto 2015). Also, there is a consensus that the decline in the capacity of the proteostasis network is one of the main drivers of aging (Hartl 2016). In most cells and tissues aging is associated with extensive loss of proteostasis equilibrium, including widespread changes in translation, a generalized downregulation of chaperones, loss of function in protein degradation machineries and impaired UPR activation (Powers and Balch 2013, Vilchez, Saez et al. 2014, Riera, Merkwirth et al. 2016). For instance, expression of almost one third of the chaperome network, including HSP40, HSP70, HSP90 and TRiC has been shown to decline during human brain aging (Labbadia and Morimoto 2015). Ultimately, this will result in accumulation of misfolded and aggregated proteins, which demolish cellular function due to their aberrant interactions with different cellular compartments (Hartl 2016). For instance, mutant huntingtin, tau or α-synuclein that underlie the pathology in HD, AD and PD respectively, has been shown to have an inhibitory effect on the protease activity on the 26S proteasome (Wong and Cuervo 2010).

Conversely, almost all signaling pathways that have been found to regulate longevity do so by modulating the proteostasis capacity (Hartl 2016). Reduction of insulin/IGF-1 signaling (IIS) is one of the most efficient means of lifespan extension and has been shown to be effective in both invertebrates and vertebrates (López-Otín, Blasco et al. 2013, Vilchez, Saez et al. 2014). Reduced IIS signaling activates the FOXO transcription factors by altering the phosphorylation state, so that the activated transcription factor can translocate to the nucleus and regulate the expression of genes that extend lifespan (Vilchez, Saez et al. 2014). The longevity extension by reduction in IIS in *C. elegans* is dependent on HSF-1 and an E3

ubiquitin ligase complex CUL-1 and suppresses age dependent aggregation and toxicity in worm models of HD and AD (Labbadia and Morimoto 2015, Hartl 2016). HSF1A, which activates HSF1 by reducing CCT activity and blocking the interaction between CCT and HSF1 has been shown to suppress toxicity in cellular models of HD (Labbadia and Morimoto 2015).

Moreover, autophagy has been demonstrated to be essential for the lifespan extension observed in all the long lived *C. elegans* strains (Vilchez, Saez et al. 2014). In addition, various lines of research provide examples of improving proteostasis by means of genetic manipulation that are enough to delay aging and decrease the incidence of age-associated pathologies in model organisms including mammalian systems (Zhang and Cuervo 2008). For instance, upregulation of autophagy has been demonstrated to ameliorate the protein toxicity in HD models of neurodegeneration (Chen, Retzlaff et al. 2011) and overexpression of chaperones extends lifespan in different model organisms (López-Otín, Blasco et al. 2013). Similarly, ectopic induction of UPS, UPR^{ER} or UPR^{mt} activity has been shown to prevent age-related decline of proteostasis and delay the onset of pathologies (Vilchez, Morantte et al. 2012, Powers and Balch 2013). Apart from genetic manipulation, regulation of PN by means of small molecules has been proven to be successful. Spermidine, an autophagy activator which in contrast to other autophagy inducers such as rapamycin does not have immunosuppressive side effects, has been shown to extend lifespan in yeast, flies and worms (López-Otín, Blasco et al. 2013).

Together, an increasing number of experiments suggest that targeting the PN by means of genetic manipulation or small molecules could hold a great promise for developing new therapies against aging and age-associated diseases.

1.3 Stem cells and stem cell proteostasis in aging

1.3.1 Stem cells are pluripotent and self-renewing

Stem cells are generally characterized by their pluripotency, the capability to differentiate into more specialized cells, and their self-renewal capacity (Schilders, Eenjes et al. 2016). A distinction can be made between embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells. ESCs are derived from the inner cell mass (ICM) of a blastocyst, a structure formed after multiple mitotic cell divisions of the zygote (Vazin and Freed 2010). A blastocyst consists of two cellular layers: the outer layer, also

known as trophoblast forms the extra-embryonic tissues such as the placenta, chorion and the umbilical cord (Vazin and Freed 2010). The ICM contains all the cells, which will give rise to the embryo, and the primitive endoderm (Nishikawa, Jakt et al. 2007, Vazin and Freed 2010). ESCs can be obtained from ICM under specific culture conditions, and possess the ability to differentiate into ectodermal, mesodermal and endodermal lineages (Nishikawa, Jakt et al. 2007, Schilders, Eenjes et al. 2016).

ESCs can maintain their pluripotency and functional confirmation of this characteristic can be achieved by analysis of expression levels of genetic markers of pluripotency or different germ layers, or formation of teratoma in *vivo* (Vazin and Freed 2010). Their capability of resuming development once seeded back into the blastocyst has made ESCs a popular tool as a vehicle to introduce genetic manipulations in model organisms (Martello and Smith 2014).

Although ESCs might acquire chromosomal aberrations due to prolonged maintenance in *vitro*, they do not undergo senescence and exhibit an indefinite proliferative lifespan (Martello and Smith 2014). This great self-renewal capacity, together with their differentiation potential makes ESCs a valuable tool for studying embryogenesis and development, as well as disease modelling, drug discovery and regenerative medicine (Nishikawa, Jakt et al. 2007, Schilders, Eenjes et al. 2016). This is particularly important for studying human biology, where availability of tissue sample is extremely limited. There are, however, ethical issues that can hamper the use of ESCs because these cells are derived from embryos produced by in vitro fertilization for clinical purposes. Immune incompatibility is the other constraint for using ESCs in regenerative medicine.

The ground breaking discovery that differentiated cells can be reprogrammed to pluripotent state resulting to formation of induced pluripotent stem cells (iPSCs) by expression of four transcription factors OCT4, SOX2, KLF4 and MYC (Takahashi and Yamanaka 2006) was a major step forward in using the promising potential of stem cells. iPSCs resemble ESCs in different aspects of their biology from morphology, regulation of signaling pathways and the capability to differentiate towards all the three primary germ layers, as well as forming teratomas *in vivo* (Medvedev, Shevchenko et al. 2010). Strikingly, iPSCs created from adult somatic cells were shown to be able to develop to viable, fertile organisms in mice (Zhao, Li et al. 2009, Zhao, Li et al. 2010). The process of reprogramming,

however, is just beginning to be understood. There are many methods for inducing pluripotency, and it seems that not all the abovementioned transcription factors are essential for reprogramming of all cell lines. For instance, fibroblasts have been shown to be able to reprogram without expression of MYC, or neural stem cells only require OCT4 expression to be reprogrammed (Jopling, Boue et al. 2011). Moreover, the efficiency of the process is still very low, and it takes a substantial period of time to induce pluripotency (Jopling, Boue et al. 2011). There are also different studies which suggest that reprogramming process demonstrates significant variability, which leads to aberrant epigenomic reprogramming, which can result in different gene expression signatures compared to ESCs (Chin, Mason et al. 2009, Lister, Pelizzola et al. 2011). Taken together, although iPSCs provide a great potential to be used as a tool for understanding development and disease, a much deeper understanding of iPSC biology is necessary before application of ESCs could be wholly substituted with iPSCs.

1.3.2 Stem cell exhaustion contributes to organismal aging

Beside ESCs and iPSCs, adult stem cells are a group of stem cells, which reside in different tissues throughout the lifetime, and are responsible for the repair and regeneration in that tissue. In human body, these include hematopoietic stem cells (HSCs), intestinal stem cells, mammary stem cells, olfactory stem cells, mesenchymal stem cells, endothelial stem cells and neural stem cells. Adult stem cells are capable of differentiating into multiple cell types of a tissue, but exhibit a lower level of pluripotency and self-renewal compared to ESCs (Schilders, Eenjes et al. 2016). There is a consensus that stem cell exhaustion, which underlies the decline in regenerative potential of tissues, is one of the main drivers of aging (Ho, Wagner et al. 2005, López-Otín, Blasco et al. 2013). Signs of aging could be detected in stem cells of different tissues such as hematopoietic stem cells, intestinal stem cells, satellite cells, neural or skin stem cells (Schultz and Sinclair 2016). This phenotypes are mainly considered to be a result of accumulated DNA damage, cell cycle dysregulation and telomere shortening (López-Otín, Blasco et al. 2013). Additionally, as stem cells rely on the signals from the environment, or niche, aging of the niche or inflammation can also interfere with the cellular communication and thus impair stem cell function (Schultz and Sinclair 2016).

As any damage in the stem cells could influence the resulting lineage of cells, this accumulation of damage in stem cells is potentially more harmful for the organism. In order

to counteract the detrimental consequences, stem cells use different strategies such as asymmetric segregation of damaged cellular components and enhanced proteostasis. Stem cells have the capacity of undergoing two kinds of cell divisions: symmetric division leads to production of two daughter cells with equivalent biology, whereas asymmetric division produces a daughter cell and a differentiating cell (Martello and Smith 2014). Similar to budding yeast, stem cells asymmetrically segregate damaged organelles and proteins to the differentiating cell, so that the daughter stem cell, which will presumably last longer can be kept in a young state (Schultz and Sinclair 2016). This damage clearance is so essential for the function of stem cells, that if disrupted, it can lead to complete loss of stem cell properties in the progeny cells (Katajisto, Döhla et al. 2015).

1.3.3 Adopting stem cell proteostasis as a novel paradigm for developing therapies against aging and age-related diseases

Stem cells, as described above, demonstrate striking differences in their biology compared to somatic cells. One of the major differences is at the level of proteostasis mechanisms. Components of the chaperome network, for instance, are differentially regulated in stem cells. At the transcript level, 119 genes were found to be downregulated and 44 genes were upregulated during differentiation of hESCs to neural progenitor cells (NPCs). At the protein level, 36 out of 122 identified chaperome components decrease during differentiation of hESCs into NPCs. In contrast, 27 chaperome components were increased during neural differentiation (Noormohammadi, Khodakarami et al. 2016). Some of the HSPs seem to have specialized functions in stem cells, which makes them indispensable for stemness. For instance, HSP90 interacts with OCT4 and NANOG and protects them from degradation by the UPS and downregulation of HSP90 leads to differentiation of mESCS (Vilchez, Simic et al. 2014).

It has been shown that mouse and human ESCs exhibit higher proteasome activity compared to their differentiated counterparts such as neurons, fibroblasts and trophoblasts (Vilchez, Boyer et al. 2012). This increased proteasome activity is essential for stem cell pluripotency and reprogramming, and is dependent on higher levels of the 19S proteasome subunit PSMD11, which in turn stabilizes the interaction of the 20S and 19S. Other proteasome subunits such as α2–α5 and the immunoproteasome subunits β1i and β5i are also increased in hESCs (Vilchez, Saez et al. 2014). Interestingly, mESCs induce the proteasome

activator PA28 to degrade damaged proteins upon initiation of differentiation and cell fate decision (Hernebring, Brolén et al. 2006).

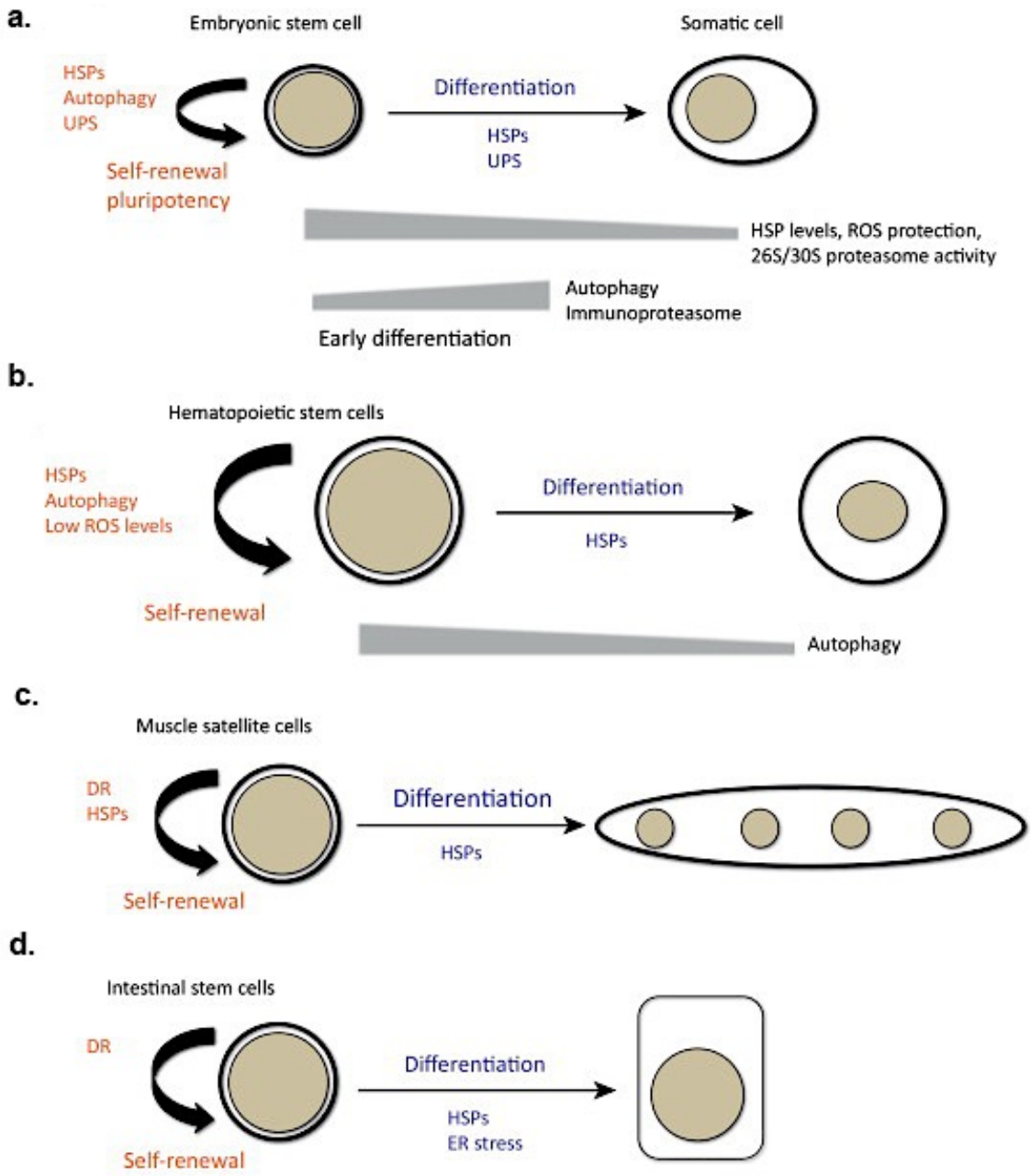

Figure V. Proteostasis of stem cells. Proteostasis in stem cells. **(A)** Embryonic stem cells (ESCs) exhibit increased levels of heat-shock protein (HSPs) and 26S/30S proteasome activity and are more protected from reactive oxygen species (ROS) than their differentiated counterparts. In an active rejuvenation step, both autophagy and the immunoproteasome activities increase during the first days of differentiation. HSPs, autophagy, and the ubiquitin–proteasome system (UPS) are required to maintain ESC features such as self-renewal and pluripotency. HSPs and the UPS are required for differentiation of ESCs into specific cellular lineages. **(B)** Hematopoietic stem cells (HSCs) exhibit increased levels of autophagy activity compared with their differentiated counterparts. Low levels of ROS and increased levels of HSPs and autophagy activity are required to maintain HSC self-renewal. HSPs are required for differentiation of HSCs. **(C)** Dietary restriction (DR) improves muscle satellite cell self-renewal. HSPs are required for differentiation of muscle satellite cells. **(D)** DR and increased levels of HSPs improve intestinal stem cell (ISC) self-renewal. Endoplasmic reticulum (ER) stress and HSPs affect differentiation of ISCs. Image reprinted from (Vilchez, Simic et al. 2014), with permission from Elsevier.

Moreover, different stem cells such as human mesenchymal stem cells (hMSCs), HSCs, dermal and epidermal stem cells and iPSCs have been shown to have increased autophagy compared to differentiated cells, and HSCs and hMSCs were shown to be dependent on autophagy for maintaining pluripotency and proliferation (Salemi, Yousefi et al. 2012, Signer and Morrison 2013). Conversely, upon differentiation of neural or cardiac stem cells, autophagy increases and blocking autophagy impairs their differentiation (Vázquez, Arroba et al. 2012, Zhang, Liu et al. 2012)

Apart from protein clearance mechanisms, hESCs were shown to accumulate less ROS, presumably due to higher antioxidant defence mechanisms such as glutathione/thioredoxin system enzymes (Tgr, Gpx2/3/4, Gsta3, Prdx2, Pdh2), which are highly expressed in ESCs compared with their differentiated counterparts (Vilchez, Simic et al. 2014). The same is valid for components of stress response pathways such as UPR^{ER}. Components of this pathway such as IRE1, XBP1 and CHOP have been shown to have an important roles during differentiation (Vilchez, Simic et al. 2014). Additionally, ESCs not only exhibit accelerated proliferation rates, but also enhanced global translation rates compared to differentiated cells (You, Park et al. 2015, Lee, Gutierrez-Garcia et al. 2017). Decreasing translation rates by means of chemical inhibitors or genetic manipulation has been shown to affect pluripotency and reprogramming potential (You, Park et al. 2015). Thus, this enhanced proteostasis could be essential for stem cells in order to be able to cope with their higher metabolic demands. Taken together, although different types of stem cells exhibit differences in their proteostasis in different stages (Figure V) it seems that stem cells benefit from a unique proteostasis profile that helps them in responding fast to environmental stress and also provide the progeny cells with a healthy proteome.

There is a growing body of evidence that proteostasis of stem cells could serve as a model for studying aging and associated pathologies. For instance, overexpression of rpn-6, the *C. elegans* orthologue of PSMD11, was enough to enhance worm survival under proteotoxic stress conditions (Vilchez, Morantte et al. 2012). This leads to the intriguing hypothesis that it might be possible to adopt mechanisms that underlie stem cell immortality, such as enhanced proteostasis, to improve aging associated phenotypes and pathologies at the organismal level. The increased proteasome activity and the longevity phenotype in the worm is modulated by DAF-16, the worm orthologue of FOXO transcription factors (Vilchez, Morantte et al. 2012). Strikingly, FOXO4 is necessary for the increased proteasome activity in

hESCs and their differentiation into neural lineages (Vilchez, Boyer et al. 2013). FOXO1 is also required for hESC pluripotency (Zhang, Yalcin et al. 2011). Thus, FOXO transcription factors seem to be a link between stem cell function and organismal longevity.

Taken together, studying proteostasis of stem cells we will not only lead towards a deeper understanding of stem cell biology, but learning from this immortal units of life might provide a novel paradigm for development of new remedies against aging.

2 Material and methods

2.1 Material

Table 1 List of Cells, Bacteria and Worm strains

Name	Description	Reference
H9 (WA09)	hESC	WiCell Research Institute
H1 (WA01)	hESC	WiCell Research Institute
hFIB2-iPS4	HD iPSCs control	(Park, Zhao et al. 2008)
HD-iPSC#1	HD iPSC Q71	(Park, Arora et al. 2008)
ND42242	iPSC Q21 control	Coriell Institute
ND36997	iPSC Q33 control	Coriell Institute
ND41656	HD iPSC Q57	Coriell Institute
ND36998	HD iPSC Q60	Coriell Institute
ND42229	HD iPSC Q71	Coriell Institute
ND36999	HD iPSC Q180	Coriell Institute
GM02183	Fibroblast Q33 control	Coriell Institute
GM03621	Fibroblast Q60	Coriell Institute
GM04281	Fibroblast Q71	Coriell Institute
GM09197	Fibroblast Q180	Coriell Institute
ND30014	Fibroblast Q21 control	RUDCR infinite biologics
ND33392	Fibroblast Q57	RUDCR infinite biologics
DH5α	Chemocompetent *E. coli*	Thermo Fisher Scientific
DH10ß	Chemocompetent *E. col*	Thermo Fisher Scientific
HT115	RNAi strain	CGC
N2	Wild type C. elegans strain	CGC
CF512	fer-15(b26)II;fem-1(hc17)IV	CGC
CB4037	glp-1(e2141) III	CGC
DA1116	eat-2(ad1116) II	CGC
CF1041	daf-2(e1370) III	Kenyon lab
DVG9	N2, ocbEx9[myo3p::gfp]	This work
DVG20	N2,ocbEx20 [sur5p::C14C10.5, myo3p::gfp]	This work
DVG22	N2,ocbEx22 [sur5p::C14C10.5, myo3p::gfp]	This work
DVG41	N2, ocbEx41[psur5::cct-5 pmyo3::gfp]	This work
DVG44	N2, ocbEx44[psur5::cct-7, pmyo3::GFP]	This work
DVG47	N2, ocbEx47[psur5::cct-2, pmyo3::GFP]	This work
DVG48	N2, ocbEx48[psur5::cct-8, pmyo3::GFP]	This work
DVG49	N2, ocbEx49[psur5::cct-8, pmyo3::GFP]	This work
DVG50	N2, ocbEx50[psur5::cct-2, pmyo3::GFP]	This work
DVG55	AM716 x DVG9	This work
DVG57	AM716 x DVG49	This work
DVG58	N2, ocbEx55[psur5::cct-5, pmyo3::GFP]	This work
DVG59	AM716 x DVG50	This work
AM716	(rmIs284[pF25B3.3::Q67::YFP]	Morimoto lab

Table 2 List of chemicals

Name	Reference
2-Mercaptoethanol	Sigma
2-Propanol	Sigma
5-Bromo-2`Deoxyuridine	Sigma
Aceton	Sigma
Adenosine 5′-triphosphate disodium salt hydrate	Sigma
Albumine from Bovine Serum	Sigma
Ammonium Bicarbonate	Sigma
Ammoniumsulfate	Carl Roth
Aprotinin From Bovine Lung	Sigma
Bacto Yeast Extract	BD
Bacto-Tryptone	BD
Calcium Chloride Dihydrate	Sigma
Chloroform	Sigma
Cholesterol (Cholesterol) 94%	Sigma
Citric Acid	Sigma
Coomassie Brillantblau G-250	VWR
Cycloheximide	Sigma
D-(+)-Galactose	Sigma
D-(+)-Glucose	Sigma
Deoxycholic Acid Sodium	Sigma
Dibutyryl Camp Sodium Salt	Sigma
Diethyl Pyrocarbonat	Sigma
Dimethl Pimelimidate Dihydrochloride	Sigma
Dl-Dithiothreitol	Sigma
DMSO	Sigma
EDTA	Sigma
Edta Solution Ph 8,0	VWR
Ethanol 99,5% (Denat. 1% Mek)	VWR
Ethanol Pure	Sigma
Ethanolamine Free Base	Sigma
Formaldehyde, 10%, Methanol Free	Polyscience
Gelatin From Porcine Skin	Sigma
Glacial Acetic Acid	Sigma
Glycerol, 500 Ml	LifeTechnologies
Glycin	Sigma
Hepes Steril 1M Ph7.3	VWR
Hydrochloric Acid	Sigma
Hydrogen Peroxide 30%	Sigma
Igepal Ca-630	Sigma

Isopropanol	Sigma
Kaliumhydrogenphospat	Roth
Kanamycin Sulfate	LifeTechnologies
L-Ascorbic Acid,	Sigma
L-Ascorbic Acid, Reagent Grade	Sigma
LB Broth	Merck
Magnesium Chloride Hexahydrate	Millipore
Methanol	Sigma
Methylviologen Hydrat 98% (Paraquat)	VWR
Mops	Sigma
Mowiol	Carl Roth
N-Acetyl-L-Cysteine	Sigma
N-Ethylmaleimide	Sigma
N,N-Dimethylformamid	Sigma
Nonfat-Dried Milk From Bovine	Sigma
Peptone	Sigma
Phenol Chloroform	BD
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Poly-L-Ornithine Hydrobromide	Sigma
Ponceau S Solution	Sigma
Potassium Acetate	Sigma
Potassium Chloride	Sigma
Potassium Dihydrogen Phosphate	Fisher
Potassium Hydrogen Phosphate Dibasic	ALFA
Potassium Hydroxide	ALFA
Proteinase K, Recombinant	Sigma
Rnasezap	Roche
Rubidium Chloride	Sigma
SDS- sodium dodecyl sulfate	Sigma
Sodium 4-Phenylbutyrate	Sigma
Sodium Acetate, Anhydrous	VWR
Sodium Azide	Sigma
Sodium Azide	Sigma
Sodium Chloride	VWR
Sodium Fluoride	Sigma
Sodium Hydrogen Phosphate Dibasic	Sigma
Sodium Hypochlorite Solution	ALFA
Sodium Orthovanadate	Sigma
Sodium Selenite	Sigma
Sucrose	Sigma
Terrific Broth	Sigma
Tetracycline Hydrochloride	VWR
Thapsigargin	Sigma
Transferin, Human, Recombinant	Sigma
	Sigma

Trichloroacetic Acid	Sigma
Tris 1M Ph 7,5	VWR
Triton X-100	Sigma
Trizma Base	Sigma
Trypan Blue Solution (0,4%)	Sigma
Tunicamycin	Sigma
Tween® 20	Biochemica

Table 3 List of culture media and chemicals

Name	Source
10X dPBS	Life technologies
10X Tris Buffered Saline	BioRad
10X Tris/Boric Acid/Edta	BioRad
10X Tris/Glycine	BioRad
10X Tris/Glycine/Sds	BioRad
1X Pbs, W/O Ca/Mg	Life technologies
4X Laemmli Sample Buffer	BioRad
4X500Ml Acrylamide/Bis Solution 37,5:1	Serva
Accutase 100 Ml	Life technologies
Agar Agar	Serva
Agarose, Ultrapure 500G	Life technologies
Ammonium Persulfate, 10 G	BioRad
B27 Serumfree Supplement	Life technologies
BDNF Human	Peprotech
Carbenicillin, Disodium Salt	Life technologies
Cf-1 Mefs	Amsbio
Collagenase Type Iv	Life technologies
Dapi (4,6 Diaminido-2-Phenylin)	Life technologies
Depc-Treated Water	Life technologies

Dispase	Stemcell Technologies
Dmem	Life technologies
Dmem, No Glucose	Life technologies
Dmem/F-12, Hepes	Life technologies
Dmem/F:12	Life technologies
Epidermal Growth Factor	Sigma
Fbs South American Hi	Life technologies
Fgf-Basic Human 1000µg	Peprotech
Fluorsave Reagent	Merck
GDNF Human	Peprotech
Geltrex Ldev	Life technologies
Gentle Cell Dissociation Reagent	Stemcell Technologies
Glutamax 1	Life technologies
Glycogen	Life technologies
Hanks' Balanced Salt Solution	Sigma
Hoechst	LifeTechnologies
Incuwater-Clean	VWR
Iptg (Dioxane Free) 50G	Promega
Knockout Serum Replacement	Life technologies
Laminin	Life technologies
Non Essential Amino Acids	Life technologies
Minimum Essential Eagle Medium	Sigma
mTeSR1	Stemcell Technologies
N2 Supplement	Life technologies
Neurobasal Medium	Life technologies
Nitrocellulose Membrane 45µm	Bio-Rad
Optimem	Life technologies
Pbs, W/O Ca/Mg (10X)	Life technologies
Penicilin/Streptomycin	Life technologies
Protease Inhibitor (25 Tabl.)	Sigma
Puromycin10X1Ml	Life technologies
Rock Inhibitor (Y-27632 Dihydrochloride)	Abcam
SDS Solution 20% (w/v)	BioRad
Sodium Bicarbonate	Life technologies
Stacking Gel Buffer	BioRad
Stemdiff Definitive Endoderm	Stemcell Technologies
Stemdiff Neural Induction Medium	Stemcell Technologies
Stemdiff Neural Progenitor Medium	Stemcell Technologies
Trypsin 0.25 Edta	Life technologies
Yeast Extract	BD

Table 4 List of chemicals and enzymes for molecular biology

Name	Source
EcoRI-HF®	NEB
NheI-HF®	NEB
NotI-HF®	NEB
XbaI	NEB
XmaI	NEB
Gibson Assemblytm Master Mix	NEB
T4 Polynucleotide Kinase	NEB
T4 Polynucleotide Kinase	NEB
T4 Dna Ligase	NEB
T4 Dna Ligase	NEB
T7 Endonuclease I	NEB
Dna Pol. Large Fragment	NEB
T4 Rna Ligase 2, Truncated	NEB
Mung Bean Nuclease	NEB
Taq Dna Polymerase	NEB
Exonuclease I	NEB
Rnase H, Recombinant	NEB
Dnase I (Rnase-Free)	NEB
Quick Ligation Kit	NEB
Deoxynucleotide Solutions, Mix	NEB
Quick-Load® 1 Kb Dna Ladder	NEB
Q5® High-Fidelity Dna Polymerase	NEB
Onetaq® Dna Polymerase	NEB
Peqgreen	Peqlab
1Kb Plus Dna Ladder 250µg	Fisher Scientific
Blue/Orange Loading Dye	Promega
Taq Polymerase	VWR
Shrimp Alkaline Phosphatase	NEB
Plasmid-Safe Dnase	Biozym
Serva Fastload 100bp DNA ladder	Serva
Dnase I (Rnase-Free)	Fisher Scientific
Ribonucleoside-Vanadyl Complex	NEB
Rnase Out Rnase Inhibitor	LifeTechnologie
Trizol Ls 200Ml	Life technologies
Ultrapure Dnase/Rnase-Free Water	Life technologies

Table 5 List of Antibodies and chemicals for protein biochemistry

Name	Source
Anti-Polyglutamin	Proteintech
Alexa Fluor 488 Goat anti Mouse IgG (H+L)	Life technologies
Alexa Fluor 488 Goat anti Rabbit IgG (H+L)	Life technologies
Alexa Fluor 546 Goat anti Mouse IgM	Life technologies
Alexa Fluor 568 F(ab')$_2$ Fragment of Goat Anti-	Life technologies
Anit-Map2	Sigma
Anti-PSME4	Abcam
Anti-CCT6a	Abcam
Anti-CCT8	BioZol
Anti-ECM29 antibody	Abcam
Anti-Nanog	Stemcell technologies
Anti-TCP1 alpha	Abcam
Anti-TCP1B	Abcam
Anti-TCP1E	Abcam
HRP AP Donkey Anti-Mouse IgG (H+L)	Jackson Immuno Research
HRP AP Donkey Anti-Rabbit IgG (H+L)	Jackson Immuno Research
HRP AP Donkey Anti-Rat IgG (H+L)	Jackson Immuno Research
Stemdiff Neural Progenitor Antibodypanel	Stemcell Technologies
Clarity Western ECL Substrate	BioRad
Hi-Mark Pre-Stained Proetin Ladder, 250µl	Life technologies
Immobilon Western Chemilum Hrp Substrate	Merck
PVDF.45Um Mmobilon	Merck
Mini-Protean TGX 10%	Bio-Rad
Precision Plus Protein Dual Color Standards, 5X	BioRad
Protein Assay Dye Reagent Concentrate, 450 Ml	BioRad
Resolving Gel Buffer, 1.5 M Tris-Hcl, Ph 8.8, 1	BioRad
Filter Trap Membrane	VWR
Temed	BioRad
Tris	BioRad

Table 6 List of kits

Name	Source
Addgene FireLab Kit	Addgene
Fugene	Promega
HiSpeed Plasmid Maxi Kit	Quiagen
Normocin	InvivoGen
Pierce BCA Proetin Assay Kit	Fisher Sientific
Puerlink Mini Kit	Life Technologie
Qiagen LongRange 2Step RT-PCR Kit	Quiagen
Qiagen Plasmid Plus Midi Kit	Quiagen
Qiagen-tip 20	Qiagen
QIAquick PCR purification Kit	Quiagen
QIAzol Lysis Reagent	Quiagen
Quanta Q Flex Reverse Transcription Kit	VWR
RNA Bee	Gentaur
SsoAdvanced SYBR Green Supermix	Bio-Rad
StemTag Alkaline Phospate Staining Kit	BioCat
Zymoclean Gel DANN Recovery Kit	Zymo

Table 7 List of plasmids

Name	Description	Reference
CCT8 ORF HA	Mammalian CCT8 ORF	Sino Biological
pDV032	Sur5p::C14C10.5 (*PSME4* orthologue)	This work
pDV066	Sur5p::T21B10.7.1 (*CCT-2* orthologue)	This work
pDV061	Sur5p::C07G2.3 (*CCT*-5 orthologue)	This work
pDV064	Sur5p::T10B5.5 (*CCT-7* orthologue)	This work
pDV067	Sur5p::Y55F3AR.3a (*CCT-8* orthologue)	This work
pDV068	Sur5p::F01F1.8a.1 (*CCT-6* orthologue)	This work
pDV077	GFP under *cct-8* promoter	This work

Table 8 List of Lentivirus

Name	Source
No-target Control (SHC016V-1EA)	Sigma
PSME4 (TRCN00000158223)	Sigma
PSME4 (TRCN00000152425)	Sigma
CCT2 (TRCN0000029499)	Sigma
CCT2 (TRCN0000029500)	Sigma
CCT6a (TRCN0000062514)	Sigma
CCT6a (TRCN0000062515)	Sigma
CCT7 (TRCN0000147373)	Sigma
CCT7 (TRCN0000149108)	Sigma
CCT8 (TRCN0000438803)	Sigma
CCT8 (TRCN0000442235)	Sigma

Table 9 List of primers used for molecular cloning

Name	Sequence (5´-3´)
C14C10.5 NotI F	TTGGCGGCCGCACTATCTTCCCATTGCAGTTATTTT
C14C10.5 XbaI R	CAATCTAGACCGAAAGGGTTGTGGGATGA
T21B10.7.1 NheI F	TTGGCTAGCATGCTTCCAGTCCAAATTTTAAA
T21B10.7.1 NotI R	CAAGCGGCCGCTTAGCATGGTCGGTTATCTTG
C07G2.3a.1 NheI F	TTGGCTAGCATGGCGCAGTCATCTGC
C07G2.3a.1 NotI R	CCAGCGGCCGCTTAATATCCCATTCTTTCATCGTCA
F01F1.8a.1 NheI F	TTGGCTAGCATGTCGTCAATCCAGTGCCT
F01F1.8a.1 NotI R	CCAGCGGCCGCTTATTCTGGTTGTGGCTGCTTC
T10B5.5a NheI F	TTGGCTAGCATGATGCGCCCACCAATTAT
T10B5.5a NotI R	CAAGCGGCCGCTTATTGCCCGGGAAGTCCTG
Y55F3AR.3a NheI F	TTGGCTAGCATGGCTATGAAGATCCCAAAGTC
Y55F3AR.3a EcoRI R	CAAGAATTCTTAGGCCATTCCATCGTCATC
Y55F3AR.3a promoter F XmaI	TTGTCTAGATTAGGCCATTCCATCGTCATC
Y55F3AR.3a promoter R XbaI	TTGCCCGGGATGGCTATGAAGATCCCAAAGTC

Table 10 List of primers used for qPCR

Name	Forward (5′-3′)	Reverse (5′-3′)
ACTB	CTGGCACCCAGCACAATG	CCGATCCACACGGAGTACTTG
GAPDH	GCACCGTCAAGGCTGAGAAC	GGATCTCGCTCCTGGAAGATG
0CT4	GGAGGAAGCTGACAACAATGAAA	GGCCTGCACGAGGGTTT
NANOG	AAATCTAAGAGGTGGCAGAAAAACA	GCCTTCTGCGTCACACCATT
SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
DPPA4	CTGGTGCCAACAATTGAAGCT	AGGCACACAGGCGCTTATATG
DPPA2	GTACTAATGGCAAGAAAATCGAAGTT	GCCGTTGTTCAGGGTAAGCA
ZFP42	CCTGCAGGCGGAAATAGAAC	GCACACATAGCCATCACATAAGG
AFP	GAGGGAGCGGCTGACATTATT	ACCAGGGTTTACTGGAGTCATTTC
ALB	TGAGGTTGCTCATCGGTTTAAA	GCAATCAACACCAAGGCTTTG
CD117	CCAAGGCCGACAAAAGGA	GGCGGGAGTCACATCTCTTTC
CXCR4	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
GATA4	TCCGTGTCCCAGACGTTCTC	GAGAGGACAGGGTGGATGGA
GATA6	AGCGCGTGCCTTCATCA	GTGGTAGTTGTGGTGTGACAGTTG
PECAM	GGAGTCCAGCCGCATATCC	GCTTGGAAAATAGTTCTGTTATGTTC
SOX17	TGGCGCAGCAGAATCCA	CGACTTGCCCAGCATCTTG
FGF5	ACGAGGAGTTTTCAGCAACAAAT	TTGGCACTTGCATGGAGTTTT
NES	TGAAGGGCAATCACAACAGG	TGACCCCAACATGACCTCTG
PAX6	CATACCAAGCGTGTCATCAATAAAC	TGCGCCCATCTGTTGCT
VIM	TCTGCCTCTTCCAAACTTTTCC	AACCAGAGGGAGTGAATCCAGAT
CDH2	CAGCAACGACGGGTTAGTCA	TGCAGCAACAGTAAGGACAAACA
EOMES	ATGCAGGGCAACAAAATGTATG	GTCTCATCCAGTGGGAACCAGTA
FOXF1	AGCCGTATCTGCACCAGAACA	ACTCCTTTCGGTCACACATGCT
MSX1	CTCCGCAAACACAAGACGAAC	CACATGGGCCGTGTAGAGTC
GATA6	AGCGCGTGCCTTCATCA	GTGGTAGTTGTGGTGTGACAGTTG
SNAI2	TGCGGCAAGGCGTTTT	CTCCCCCGTGTGAGTTCTAATG
CCT2	AAGCCACGAAGGCTGCAA	TCATCGGAACCATGATCAACTG
CCT5	CGGATAAGTGCCCCACCTTA	TCCAGTGCGTCGGCAAA
CCT6A	TGGCCAGAACATCTCTTCGTACT	AGTCCACTACAGCCTCTGTTAAGAC
CCT7	GTGGCATGGACAAGCTTATTGTAG	CAGAATTGTGGCCCCATCA
CCT8	ACCCGGAGGTGGAGCAA	GGACATGTCTCTCCATATGATGTGA
cdc-42	CTGCTGGACAGGAAGATTACG	CTCGGACATTCTCGAATGAAG
pmp-3	GTTCCCGTGTTCATCACTCAT	ACACCGTCGAGAAGCTGTAGA
cct-1	CCAGCCAAGCTTGAGGCTAT	TCAATGCGGCGTTTGGTA
cct-2	GGACTTGACTCGGCTGAACTTG	CGATGTCGATTCCCATATTGTG

cct-3	GCGTGAGAGTGGCCATCAG	CGGCGATCGTCTTGCAA
cct-4	TGCACTTGAGCTCATTCCATACA	AACAGTGTGAATTGGTGACAATCC
cct-5	TCTTGGAAGCAAAATCGTAAACC	ACAGCATCGACAGCGATTTCT
cct-6	ACGAAATGGCGATTCAACATC	GTCGTCTTGTGCCGTCGAA
cct-7	TTGAGGGCAAGGATCAAGCT	CGTGGGATGATCTCGAAAGC
cct-8	CGGCCGCTTTGTTGGA	TGTGGTGTCATTCCCATATGGA
enpl-1	GAAACAAGAGAGGAAGATTCGATCA	GCTTTTGAGCGGAGCTCCTT

Table 11 List of electrical devices

Instrument	Manufacturer
BINDER CB-150	BINDER GmbH
Mini Trans-Blot Cell	Bio-Rad
Mini-Protean TGX, 10% ;10W; 10	Bio-Rad
Mini-Sub Cell GT System W/ 7x7	Bio-Rad
Mini-Trans-Blot Module	Bio-Rad
C1000 Touch Thermal Cycler	Bio-Rad
S1000 Thermal Cycler	Bio-Rad
Mini-PROTEAN Tetra Cell	Bio-Rad
PowerPac Basic HC	Bio-Rad
Eppendorf 5430R, 5424, 5810R	Eppendorf
Thermomixer Comfort	Eppendorf
Hanna Instruments HI3220	HANNA Instruments GmbH
Heidolph unimax 1010	Heidolph Instruments GmbH
ScanLaf Class 2 Mars	Labogene
VTX-3000L Mixer Uzusio	LMS Co., Ltd
Shaking platform RM S-30V	M. Zipper GmbH
INCUCELL MMM	MMM group
MIR-554-PE Cooled Incubator	Panasonic
EnSpire Multimode Plate Reader 2300	Perkin Elmer
Precisa XB4200C	Precisa Gravimetrics AG
Dri-Block DB-2D	Techne
NanoDrop 8000	ThermoFisher Scientific
Fusion Solo	Vilber Lourmat
SteREO Discovery.V8	ZEISS
ZEN imaging software	ZEISS
Imager. Z1	ZEISS
ZEISS Apotome .2	ZEISS
Ec-Plan-Neofluar 20X-40X	ZEISS
Achro-plan 10X	ZEISS
Axiocam 506 mono	ZEISS
Axio Zoom.V16	ZEISS

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Cell culture and differentiation

hESCs and iPSCs were maintained on Geltrex (ThermoFisher Scientific) using mTeSR1 (Stem Cell Technologies). hESCs and iPSCs colonies were passaged using a solution of dispase (2 mg ml^{-1}), and scraping the colonies with a glass pipette. All the cell lines used in this study were tested for mycoplasma contamination at least once every three weeks.

Neural differentiation of H9 hESCs, H1 hESCs and iPSCs was performed following the monolayer culture method, following the STEMdiff Neural Induction protocol (Stem Cell Technologies) as described previously (Chambers, Fasano et al. 2009). Briefly, undifferentiated pluripotent stem cells were rinsed once with PBS and then added with 1 ml of Gentle Dissociation Reagent (Stem Cell Technologies) for 10 min. After the incubation period, cells were dislodged in 2ml DMEM F-12 supplemented with 10 mM ROCK inhibitor (Abcam). Then cells were centrifuged at 300g for 10 min, resuspended on STEMdiff Neural Induction Medium supplemented with 10 mM ROCK inhibitor and plated on polyornithine (15 mg ml^{-1})/laminin (10 mg ml^{-1})-coated plates (200,000 cells cm^{-2}). For neuronal differentiation, NPCs were dissociated with Accutase (Stem Cell Technologies) and plated into neuronal differentiation medium (Dulbecco's Modified Eagle Medium (DMEM)/F12, N2, B27 (ThermoFisher Scientific), 1 μg ml^{-1} laminin (ThermoFisher Scientific), 20 ng ml^{-1} BDNF (Peprotech), 20 ng ml^{-1} GDNF (Peprotech), 1 mM dibutyryl-cyclic AMP (Sigma) and 200 nM ascorbic acid (Sigma)) onto polyornithine/laminin-coated plates as described in (Vilchez, Boyer et al. 2012). Cells were differentiated for 1–2 months, with weekly feeding of neuronal differentiation medium.

Endoderm differentiation of H9 hESCs was performed using STEMdiff Definitive Endoderm Kit (Stem Cell Technologies). Cardiomyocyte differentiation was performed as previously described (Rao, Pfeiffer et al. 2015). Briefly, confluent H1 hESCs were dissociated into single cells with Accutase at 37°C for 10 min followed by inactivation using two volumes of F12/DMEM. Cells were counted and 230000 cells/cm^2 where plated in ITS medium (Corning), containing 1.25 μM CHIR 99021 (AxonMedchem) and 1,25 ng ml^{-1} BMP4 (R&D), and seeded on Matrigel-coated 24-well plates. After 24 h, medium was changed to TS (transferrin/selenium) medium. After 48 h, medium was changed to TS

medium supplemented with 10 μM canonical Wnt-Inhibitor IWP-2 (Santa Cruz) for 48 h. Then, medium was changed to fresh TS until beating cells were observed at days 8 to 10. Finally, medium was changed to Knockout DMEM (ThermoFisher Scientific) supplemented with 2% FCS, L-Glutamine and Penicillin/Streptomycin until cells were used for downstream analysis.

2.2.1.2 Transfection and lentiviral infection

For transient lentiviral infections, hESC/iPSC colonies growing on Geltrex were incubated with mTesR1 medium containing 10 μM ROCK inhibitor (Abcam) for 2 h and individualized using Accutase. Hundred thousand cells were plated on Geltrex plates and incubated with mTesR1 medium containing 10 μM ROCK inhibitor for 1 day. Cells were infected with 5 μl of concentrated lentivirus. Plates were centrifuged at 800g for 1 h at 30°C. Cells were fed with fresh media the day after to remove virus. After 1 day, cells were selected for lentiviral integration using 1 μg ml^{-1} puromycin (ThermoFisher Scientific). Cells were collected for experimental assays after 4-6 days of infection. Alternatively, we generated stable transfected hESCs. In this case, hESC colonies growing on Geltrex were incubated with mTesR1 medium containing 10 μM ROCK inhibitor for 1 h and individualized using Accutase. Fifty thousand cells were infected with 20 μl of concentrated lentivirus in the presence of 10 μM ROCK inhibitor for 1 h. Cell suspension was centrifuged to remove virus, passed though a mesh of 40 μM to obtain individual cells, and plated back on a feeder layer of fresh mitotically inactive mouse embryonic fibroblasts (MEFs) in hESC media (DMEM/F12, 20% knockout serum replacement (ThermoFisher Scientific), 1 mM L-glutamine, 0.1 mM non-essential amino acids, β-mercaptoethanol and 10 ng ml^{-1} bFGF (Joint Protein Central)) supplemented with 10 μM ROCK inhibitor. After a few days in culture, small hESC colonies arose. Then, we performed 1 μg ml^{-1} puromycin selection during 2 days and colonies were manually passaged onto fresh MEFs to establish new hESC lines.

HEK293T cells (ATCC) were plated on 0.1% gelatin-coated plates and grown in DMEM supplemented with 10% FCS and 1% non-essential amino acids (ThermoFisher Scientific) at 37°C, 5% CO_2 conditions. Cells were transfected once they reached 80-90% confluency. 1 μg CCT8 overexpression plasmid (Sino Biological HG11492-UT) or pCDNA3.1 empty vector plasmid (Life Technologies V870–20) were used for transfection, using Fugene HD (Promega) following manufacturer's instructions. 24 h after transfection, the cells were treated with 500 μg ml^{-1} Hygromycin B gold (Invivogen) during 16 h to select

for transfected cells. After 24 h incubation in normal medium, the cells were harvested for TRiC/CCT assembly experiments.

2.2.1.3 Immunofluorescence

Cells were fixed with paraformaldehyde (4% in PBS) for 20 min, followed by permeabilization (0.2% Triton X-100 in PBS for 10 min) and blocking (3% BSA in 0.2% Triton X-100 in PBS for 10 min). Cells were incubated in primary antibody overnight at 4 °C. After washing with PBS, cells were incubated in secondary antibody and co-stained with 2 mg ml-1 Hoechst 33342 (Life technologies) for 1h at RT. Finally, coverslips were fixed on glass slides with Mowiol (Sigma).

2.2.1.4 Bacterial cultures

Bacteria were cultured overnight either on LB plates or liquid LB culture supplemented with the desired antibiotics (100 mg/mL Carbenicillin and/or 10µg/mL Tetracyclin). LB medium was prepared according to the following recipe, using sterile technique:

Table 12 LB medium recipe

Ingredient	Amount
Bacto-Tryptone	10g
Yeast extract	5g
NaCl	10g
H_2O	up to 1L
Bacto-Agar (for plates)	15g

2.2.2 *C. elegans* maintenance and assays

2.2.2.1 *C. elegans* cultivation and lifespan

Wild type and transgenic worms were cultured using standard methods (Brenner 1974) on NG plates seeded with OP50. Lifespan analyses were performed as described previously (Vilchez, Morantte et al. 2012). Animals were grown at 20ºC until day 1 of adulthood. 96 animals were used per condition and scored every day or every other day (Amrit, Ratnappan et al. 2014). From the initial worm population, the worms that are lost or burrow into the medium as well as those that exhibit 'protruding vulva' or undergo bagging were censored. Lifespans were conducted at either 20ºC or 25ºC as stated in the figure legends. For non-integrated lines DVG9, DVG41, DVG44, DVG47-50 and DVG58, GFP positive worms were selected for lifespan studies.

2.2.2.2 Heat stress assay

For day 1 adulthood heat-shock assays, eggs were transferred to plates seeded with *E. coli* (OP50) bacteria and grown to day 1 of adulthood at 20ºC. Worms were then transferred to fresh plates with *E. coli* (HT115) containing L4440 and heat shocked at 34ºC. Worms were checked every hour for viability. For day 5 adulthood heat-shock assays, adult worms were transferred to fresh plates with *E. coli* (HT115) containing L4440 every day and then heat shocked at 34ºC at day 5.

2.2.2.3 Oxidative stress assay

Worms were synchronized by egg laying on OP50 (in case of OE lines) or EV RNAi on normal growth plates. For RNAi experiments, worms were transferred to the RNAi bacteria at L4 stage in order to prevent interference with the development. One day prior to the experiment, paraquat was diluted to 0.25M in ddH_2O and added to the seeded plates to reach a final concentration of 7.5 mM. The plates were kept in dark. To prevent bagging, day 5 adult worms were transferred to paraquat plates, scored daily and transferred every other day to a fresh plate.

2.2.2.4 Motility Assay

Thrashing rates were determined as previously described (Brignull, Moore et al. 2006, Vilchez, Boyer et al. 2012). Animals were grown at 20ºC until L4 stage and then grown at 20ºC or 25ºC for the rest of the experiment. Worms were fed with *E. coli* (OP50) bacteria. Worms were transferred at day 3 of adulthood to a drop of M9 buffer and after 30 seconds of adaptation the number of body bends was counted for 30 seconds. A body bend was defined as change in direction of the bend at the midbody (Chai, Shao et al. 2002).

2.2.2.5 RNAi constructs

RNAi-treated strains were fed *E. coli* (HT115) containing an empty control vector (L4440) or expressing double-stranded RNAi. *cct-2*, *cct-6*, *cct-7* and *cct-8* RNAi constructs were obtained from the Vidal RNAi library. *cct-5* and *hsf-1* RNAi constructs were obtained from the Ahringer RNAi library. All constructs were sequence verified.

2.2.2.6 *C. elegans* germline and gut immunostaining

N2 and CB4037 strains were grown at 25ºC from hatching on OP50. Worms were dissected in dissection buffer (0.2% Tween 20, 1x Egg buffer, 20mM Sodium Azide) on a coverslip. Fixation was performed by adding formaldehyde buffer (1x Egg buffer, 0.2% Tween20 and 3.7% formaldehyde) and animals were frozen on dry ice between the coverslip and a poly-lysine coated slide (ThermoScientific). The coverslips were removed and the slides were placed for 1 min at -20^{o}C in methanol, then washed twice with PBS 0.1% Tween20. After blocking for 30 min in PBST 10% donkey serum, anti-TCP1 alpha (Abcam, ab90357) antibody was added (1:1000) followed by overnight incubation in a humid chamber. Anti-Rat secondary antibody (Alexa Fluor 546) was added (1:400) for 2 h at room temperature. Finally, slides were mounted with Precision coverslip (Roth) using DAPI Fluoromount-G (Southern Biotech). Anti-TCP1 alpha is profiled for use in *C. elegans* by Abcam. We validated by western blot analysis that anti-TCP1 alpha recognizes this subunit in worms at the correct molecular weight. Native gel experiments confirmed that anti-TCP1 alpha recognizes CCT1 in its monomeric form as well as part of the TRiC/CCT complex.

2.2.2.7 Nuclear staining in RNAi-treated *C. elegans*

N2 wild-type worms were grown on *E. coli* (HT115) containing an empty control vector (L4440) from hatching until adulthood. The animals were then either kept on empty vector bacteria or fed with RNAi bacteria to *cct-2* or *cct-8* until day 4 of adulthood. Animals were then harvested and washed three times in M9 buffer. After 1 h incubation in methanol, worms were washed again three times with M9 buffer and mounted on a slide using DAPI Fluoromount-G (Southern Biotech).

2.2.3 Molecular biology

2.2.3.1 Plasmid DNA preparation

2ml (mini) or 100ml (maxi) of bacterial overnight culture were pelleted at 8000g for 10 min and DNA was isolated by Purelink Quick Plasmid Miniprep (Invitrogen) or Qiagen Maxi kits following manufacturer's instructions.

2.2.3.2 Construction of *cct C. elegans* expression plasmids.

To construct the *cct C. elegans* expression plasmids, pPD95.77 from the Fire Lab kit was digested with SphI and XmaI to insert 3.6KB of the *sur5* promoter. The resultant vector was then digested with KpnI and EcoRI to excise GFP and insert a multi-cloning site containing KpnI, NheI, NotI, XbaI, and EcoRI. T21B10.7.1 (*cct-2*), C07G2.3a.1 (*cct-5*), F01F1.8a.1 (*cct-6*) and T10B5.5a (*cct-7*) were PCR amplified from cDNA to include 5' NheI and 3' NotI restriction sites using NEB Q5 high fidelity polymerase and was then cloned into the aforementioned vector. Y55F3AR.3a (*cct-8*) was amplified with 5' NheI and 3' EcoRI. All constructs were sequence verified. All the reagents were purchased from NEB and experiments were performed following the manufacturer's instructions.

2.2.3.3. Construction of *cct-8* expression reporter construct

To construct pDV077, pPD95.77 from the Fire Lab kit was digested with XbaI and XmaI. The promoter region and part of the first intron of Y55F3AR.3a (*cct-8*) was PCR amplified from N2 gDNA to include -600 to 100 and then cloned into the aforementioned vector using the same enzymes.

2.2.3.4 Transformation of *E.coli*

Competent bacteria were either purchased from Thermofisher Scientific, or prepared as previously described (Inoue, Nojima et al. 1990). The bacteria were thawed on ice, incubated for 20 min with the plasmid DNA, and then heated at 42 ºC for 30-60s. After 5 min incubation on ice, the cells were grown in SOC medium for 1h while shaking at 800 rpm and cultured on a plate with the appropriate antibiotic for ON growth at 37 ºC.

Table 13 Recipe for SOC medium

Ingredient	Amount
Bacto-Tryptone	20g
Yeast extract	5g
NaCl	2ml of 5M solution
KCl	2.5ml of 1M solution
$MgCl_2$	10ml of 1M solution
$MgSO_4$	10ml of 1M solution
Glucose	20ml of 1M solution
H_2O	up to 1L

2.2.3.5 Gene expression analysis

For human cell samples, total RNA was extracted using RNAbee (Tel-Test Inc.). cDNA was generated using qScript Flex cDNA synthesis kit (Quantabio). SybrGreen real-time qPCR experiments were performed with a 1:20 dilution of cDNA using a CFC384 Real-Time System (Bio-Rad) following the manufacturer's instructions. Data were analysed with the comparative $2\Delta\Delta C_t$ method using the geometric mean of *ACTB* and *GAPDH* as housekeeping genes. For *C. elegans* samples, total RNA was isolated from synchronized populations of approximately 2,000 adults using QIAzol lysis reagent (Qiagen). Data were analysed with the comparative $2\Delta\Delta C_t$ method using the geometric mean of *cdc-42* and *pmp-3* as endogenous control (Hoogewijs, Houthoofd et al. 2008).

2.2.3.6. Production of transgene worms by microinjection

For generation of transgenic worm strains, a DNA plasmid mixture containing 70 ng μl^{-1} of the corresponding plasmid and 20 ng μl^{-1} pPD93_97 (*pmyo-3::GFP*) was injected into the gonads of adult N2 hermaphrodite animals, using standard methods (Mello,

Kramer et al. 1991). GFP-positive F_1 progeny were selected. Individual F_2 worms were isolated to establish independent lines. Control worms DVG9 (N2, *ocbEx9[myo3p::GFP]*) were generated by microinjecting N2 worms with 20 ng μl^{-1} pPD93_97.

2.2.4 Biochemistry

2.2.4.1 Protein biochemistry

Cells were collected from tissue culture plates by cell scraping and lysed in protein cell lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% SDS supplemented with 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride and Complete mini protease) for 1h at 1,000 r.p.m. and 4°C in a Thermomixer. Samples were centrifuged at 10,000g for 15 min at 4°C and the supernatant was collected. Protein concentrations were determined with a standard BCA protein assay (Thermoscientific). Approximately 20–30 μg of total protein was separated by SDS–PAGE, transferred to nitrocellulose membranes (Whatman) and subjected to immunoblotting.

2.2.4.2 Blue native gel immunoblotting of TRiC/CCT complex.

For experiments with hESCs (H9 and H1) and their NPC counterparts, cells were collected in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol and 10% glycerol supplemented with protease inhibitor cocktail (Roche)) and lysed by passing 10 times through a 27-gauge needle attached to a 1 ml syringe. Lysate was centrifuged at 16,000g for 10 min at 4°C. 50 μg of total protein was run on a 3-13% gel in deep blue cathode buffer (50 mM Tricine, 7.5 mM Imidazole, and 0.02 % Coomassie G250) at 4°C for 3 hour at 100V and then exchange deep blue cathode buffer to slightly blue cathode buffer (50 mM Tricine, 7.5 mM Imidazole, and 0.002 % Coomassie G250) and run at 100V overnight. Proteins were then transferred to a PVDF membrane at 400 mV for 3 hours by semi-dry blotting. Extracts were also analyzed by SDS-PAGE to determine CCT subunit expression levels and loading control.

2.2.4.3 Filter trap assay

Worms were grown at 20ºC until L4 stage and then grown at 25ºC for the rest of the experiment. Day 3 adult worms were collected with M9 buffer and worm pellets were frozen with liquid N2. Frozen worm pellets were thawed on ice and worm extracts were generated by

glass bead disruption on ice in non-denaturing lysis buffer (50mM Hepes pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X100) supplemented with EDTA-free protease inhibitor cocktail (Roche). Worm and cellular debris was removed with 8000g spin for 5 min. Approximately 100 µg of protein extract was supplemented with SDS at a final concentration of 0.5% and loaded onto a cellulose acetate membrane assembled in a slot blot apparatus (BioRad). The membrane was washed with 0.1% SDS and retained Q67-GFP was assessed by immunoblotting for GFP (ImmunoKontakt, 210-PS-1GFP). Extracts were also analyzed by SDS-PAGE to determine protein expression levels.

iPSCs were collected in non-denaturing lysis buffer supplemented with EDTA-free protease inhibitor cocktail and lysed by passing 10 times through a 27-gauge needle attached to a 1 ml syringe. Then, we followed the filter trap protocol described above. The membrane was washed with 0.1% SDS and retained polyQ proteins were assessed by immunoblotting for anti-polyQ-expansion diseases marker antibody (Millipore, MAB1574). Extracts were also analyzed by SDS-PAGE to determine HTT protein expression levels.

2.2.5 Statistics

PRISM 6 software was used for statistical analysis to determine median and percentiles. For Lifespan and heat stress assays, P-values were calculated using the log-rank (Mantel–Cox) method. The P-values refer to experimental and control animals in a single experiment. For the rest of experiments, unless stated otherwise, two-tailed unpaired students' t-test was used to check for statistical significance. Error bars represent standard error of the mean (S.E.M.). (*$p<0.05$ **$p<0.01$ ***$p<0.001$)

3 Results

3.1 Investigating the impact of PSME4 on stem cell biology

3.1.1 PSME4 is not essential for stemness

In order to validate the results we obtained from our quantitative proteomics screen which demonstrated elevated expression of the proteasome activator PSME4, we compared the expression of PSME4 in H9 hESC line with H9 cells differentiated into neural progenitor cells (NPCs) and neurons. We observed that the levels of PSME4 decreases remarkably upon differentiation to NPCs and neurons (Figure 1a-b), but surprisingly increases remarkably upon differentiation towards endoderm lineage (Figure 1c). To check whether PSME4 expression is reprogrammable, we compared the levels of PSME4 in fibroblasts and induced pluripotent stem cells (iPSCs) and observed an increase in expression (Figure 4d). As PSME4 is highly increased in hESCs, we reasoned that it might play an essential role in regulating the stem cell function. In order to investigate the role of PSME4 in stemness, we established knockdown (KD) H9 cell lines, by using two distinct short hairpin RNAs (shRNA) designed to target PSME4, and a non-targeting shRNA as control. These stable cell lines demonstrated a significant decrease in PSME4 expression, both at protein and mRNA level (Figure 1e-f).

To elucidate whether and how the hESCs downregulated for PSME4 are affected, we checked for pluripotency markers such as *OCT4*, *NANOG* and *SOX2* as well as markers of the distinct germlayers such as *GATA4*, *AFP or NESTIN* to find out whether these cells show a predisposition towards any cellular lineage. As demonstrated in Figure 2, no remarkable difference was observed among PSME4 KD and control cells in expression of these markers. Although PSME4 KD cells did not exhibit any difference in expression of pluripotency markers compared to control cells, it could be hypothesized that these cells might be limited in their differentiation capabilities. In particular, the observation that PSME4 increases in endoderm progenitor cells compared to hESCs, hints towards the possibility that PSME4 might be essential for endodermal function and thus for differentiation into this lineage. In addition, we also examined PSME4 KD hESCs capability to differentiate towards ectodermal lineage.

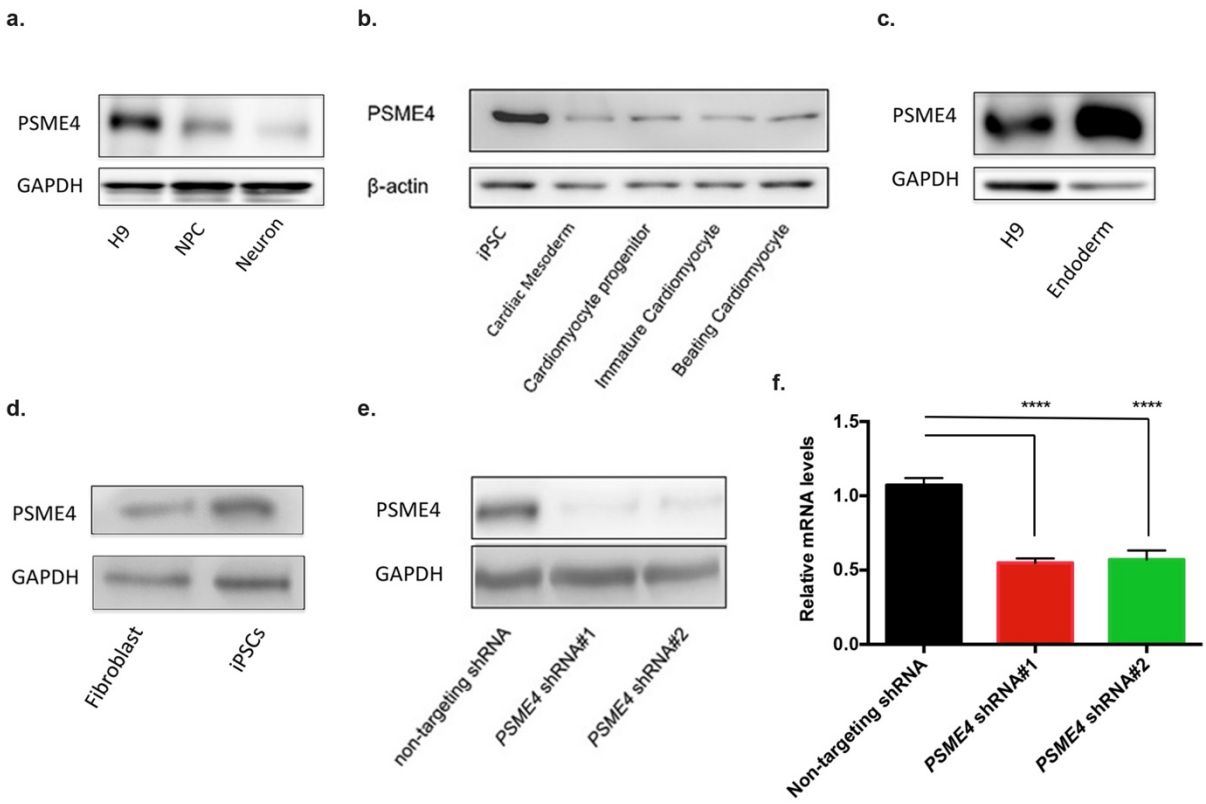

Figure 1. PSME4 is highly expressed in most of pluripotent stem cells and its expression is reprogrammable. (a-d) hESCs or iPSCs demonstrate higher PSME4 expression compared more differentiated cells of ectoderm and mesoderm lineages. Endoderm progenitor cells have increased PSME4 expression compared to hESCs. **(e-f)**. Downregulation of PSME4 in hESCs by shRNA leads to efficient knockdown. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001) (n= 3 independent experiments).

However, PSME4 KD hESCs could differentiate into either endoderm or ectoderm progenitors, without any major difference in expression of lineage specific markers. No difference was observed in the expression of endoderm markers (*GATA4*, *SOX17*, *CXCR4*, *CD117*) between PSME4KD and control cells . Although some NPC or neural markers such as *AADC*, *PAX6* or *TH* showed higher expression levels when PSME4 is downregulated, this was observed only in one of the KD lines. In addition, no significant difference could be detected in the cellular morphology after neural differentiation of PSME4 KD cells compared to the control cell line (Figure 2 c). Thus, PSME4 appears to be dispensable for maintaining the pluripotency and differentiation into endoderm and ectoderm lineages.

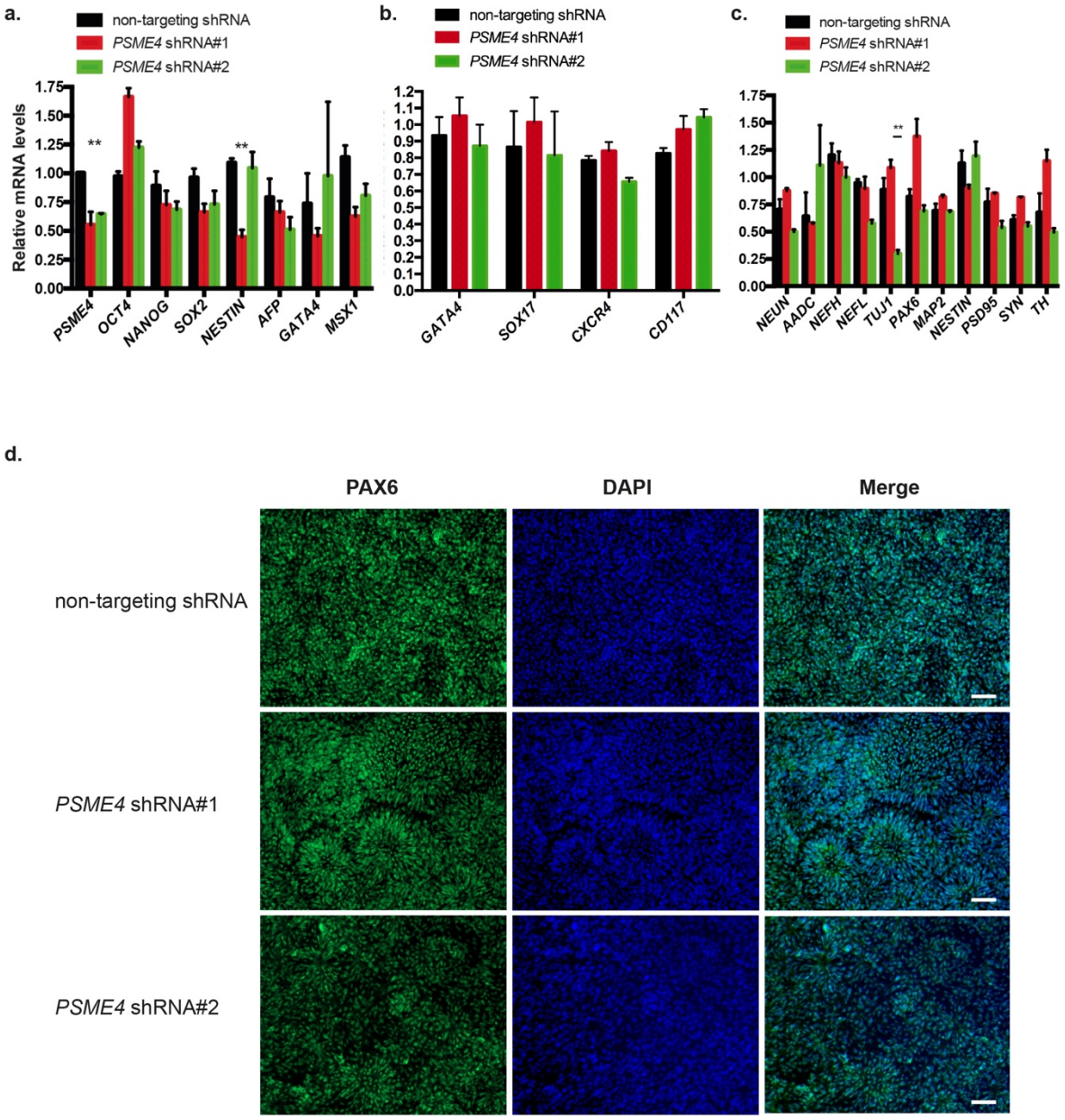

Figure 2. Lack of PSME4 does not affect the pluripotency of hESCs. (a) Downregulation of PSME4 does not lead to loss of pluripotency, as evidenced by the unaffected expression of pluripotency and lineage specific markers. **(b-c)** PSME4 KD hESCs does not exhibit any significant difference in expression of endoderm or neural differentiation markers when differentiated into these lineages **(d)** PSME4 KD cells do not exhibit any abnormalities upon differentiation into NPCs, neither morphologically, nor in expression of NPC marker PAX6 (After 6 days of neural induction, scale bar represents 50 µm). All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001) (n= 3 independent experiments).

3.2 Impact of PSME4 on longevity and stress resistance

3.2.1 psme4 does not affect longevity in *C. elegans*

As impaired proteostasis is related with a myriad of age-associated diseases and aging, and taken the relevance of stem cell proteostasis in organismal aging (Vilchez, Boyer et al. 2012, Vilchez, Morantte et al. 2012), we asked whether downregulation or overexpression of PSME4 orthologues could affect longevity in worms. *C. elegans* has two orthologues for PSME4: C14C10.5 and T28B8.3. As C14C10.5 (from now referred to as *psme4*) exhibits more similarity in the protein sequence to the mammalian gene (Figure 3a) (Goujon, McWilliam et al. 2010, Sievers, Wilm et al. 2011), we mainly concentrated on this orthologue. Worms fed with siRNA against the worm *PSME4* orthologue at 20°C (normal condition) and 25°C (mild proteotoxic stress) did not show any difference in their lifespan compared to the control worms (Figure 3b-c). We reasoned that this result might be due to inefficient RNAi, and therefore we repeated the lifespan experiment with rrf-3 strain. This strain is known to be more sensitive to RNAi due to lack of the RNA polymerase RRF-3 (Simmer, Tijsterman et al. 2002). Once again, no significant difference was observed in the longevity of control and downregulated worms (Figure 3d). In order to investigate the impact of psme4 overexpression on longevity, we created lines that overexpress psme4 in the soma of the worms (Figure 3e). This overexpression by extrachromosomal array did not have a significant impact on the longevity of the worms under normal conditions when compared to the control line (Figure 3f). This data indicates that psme4 does not affect the longevity of *C. elegans* under normal growth conditions.

a.

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T28B8.3    VYDSKWLIYMEEERKSWPTFHLGSEVPMVKTHKFASLRAHAFYILEIFVNQINVNDVDRT  20% identical to PSME4
C14C10.5   VKDEPDADG-----------------------KTYRTHVITILNSLLPGLDCNDISKC  30% identical to PSME4
PSME4      VSGGRWF--------------------------PEGPTHMLPLLMRALPGVDPNDFSKC
           * .                                :* : :*   :  :: **..:
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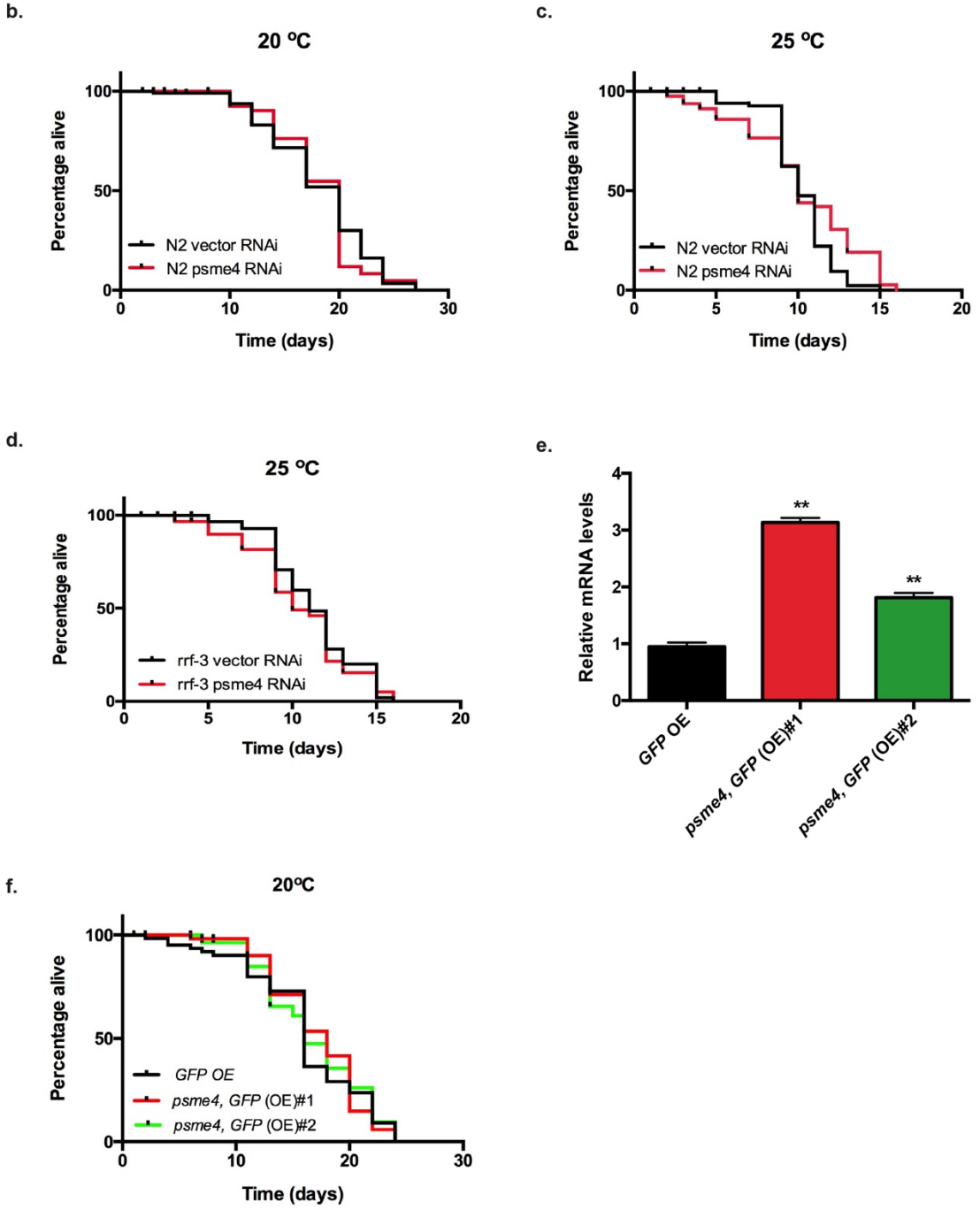

Figure 3. Misregulation of psme4 does not affect *C. elegans* longevity. **(a)** Protein alignment reveals the higher similariy of C14C10.5 to the human PSME4. Multisequence alignement was performed using Clustal Omega (EMBL-EBI). **(b-d)** Downregulation of psme4 by RNAi does not affect wildtype *C. elegans* lifespan at 20 or 25 °C, or the lifespan of RNAi sensitive rrf-3 strain. **(e)**. Somatic overexpression of psme4 by extrachromosomal array leads to significant increase of psme4 expression as demonstrated by qPCR analysis. **(f)**. Somatic overexpression of psme4 is not sufficient to extend *C. elegans* lifespan. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001) (n= 3 independent experiments).

3.2.2 psme4 mildly affects *C. elegans* stress resistance

As we could not observe any phenotypes in psme4 KD hESCs or *C. elegans*, we hypothesized that it might be dispensable for maintaining the proteostasis under normal conditions, but might be important for helping cells cope with proteotoxic stress. In order to investigate this hypothesis, we exposed the worms to two major causes of proteotoxicity, heat and oxidation, and evaluated their survival under stress conditions. Surprisingly, when the worms were exposed to heat, we observed a moderate but significant increase in the survival of worms upon downregulation, and a mild decrease in survival upon overexpression of psme4 (Figure 4a-b). In case of oxidative stress, we observed a minor, but statistically significant increase in the survival in psme4 OE worms in one of the overexpression lines (Figure 4d), whereas there were no significant differences in stress resistance of psme4 KD worms (Figure 4c-d).

Based on the mild increase in stress resistance of psme4 OE worms, we speculated that PSME4 might be also indispensable for maintenance of mammalian cells under normal conditions, but might be crucial for the capability of these cells to cope with proteotoxic stress under specific conditions or developmental stage. Intrigued by the remarkable increase in the expression of PSME4 upon differentiation towards endoderm lineage, which is in stark contrast with the expression pattern in other lineages, and drastic fluctuations in protein oxidation states during differentiation process (Hernebring, Brolén et al. 2006), we asked whether PSME4 could be essential for clearance of oxidized proteins in endoderm cells. To test this hypothesis, we checked the levels of protein carbonylation both in hESCs or their endoderm counterparts. Again, we were unable to discover any differences in the levels of protein carbonylation when PSME4 was downregulated either in hESCs or in endoderm cells (Figure 4e).

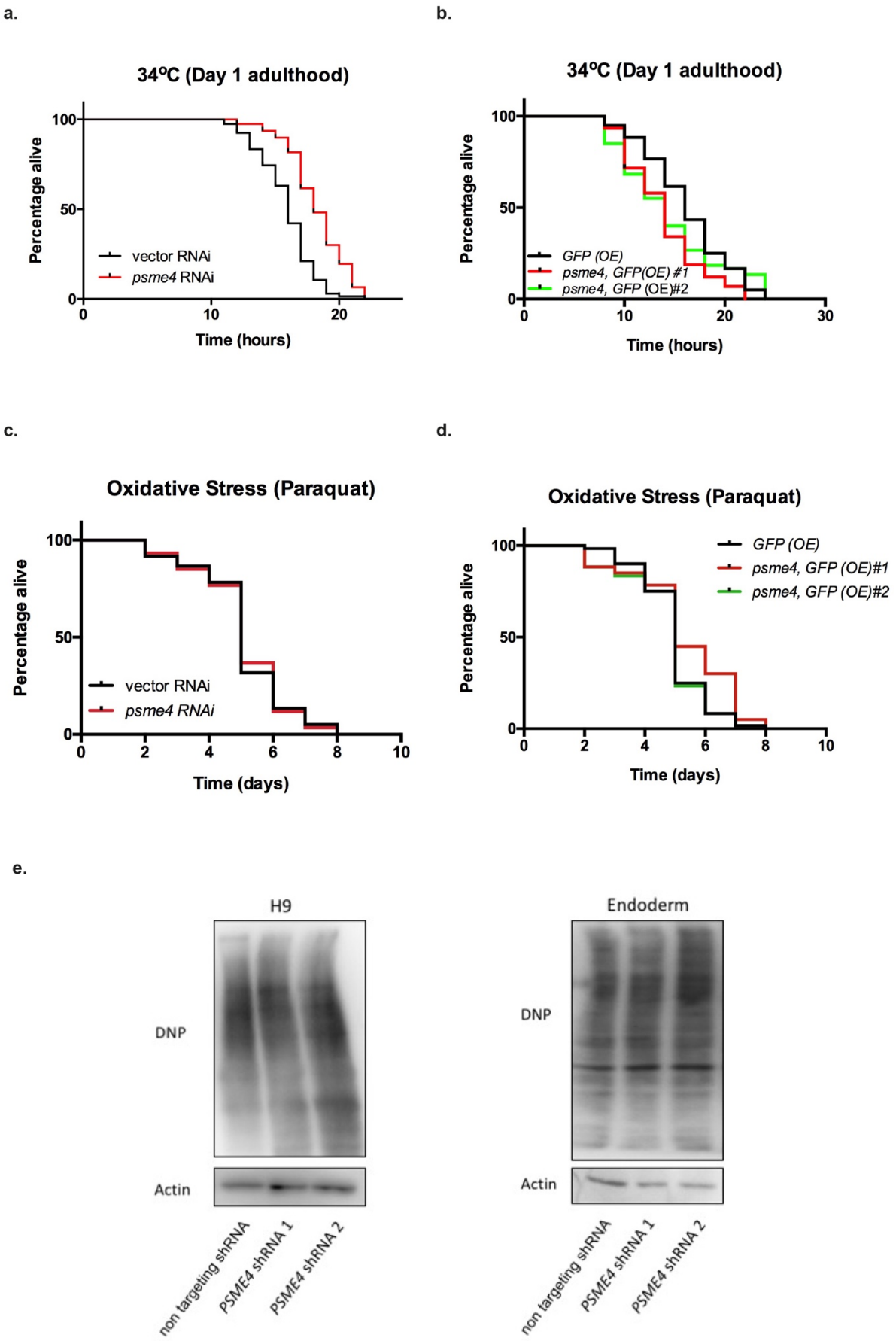

Figure 4. Misregulation of psme4 leads to minor changes in proteotoxic stress resistance of *C. elegans*. **(a)** Downregulation of psme4 increases the stress resistance of worms **(b)** psme4 OE increases the heat sensitivity of *C. elegans* (c). Downregulation of psme4 does not affect resistance against oxidative stress, whereas one of the two psme4 OE lines demonstrates a very mild increase in oxidative stress resistance **(d).** Neither hECSs nor their endoderm counterparts accumulate more oxidized proteins upon downregulation of PSME4 **(e).** All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001) (n= 3 independent experiments).

3.3 Investigating the impact of chaperome network on stem cell biology

3.3.1 CCT complex is indispensable for stem cell survival and pluripotency

3.3.1.1 CCT subunits decrease during hESC differentiation

Taken the important role of the UPS in stem cell identity, we asked whether other components of the PN could be of similar importance. To answer this, we compared these cells to their NPC and neural counterparts by quantitative mass spectrometry and RNA sequencing (Noormohammadi, Khodakarami et al. 2016). Among the major proteostasis components, we discovered the chaperome network to exhibit dramatic differences in expression in hESCs and their NPC and neural counterparts. At the protein level, we found that 36 out of 122 identified chaperome components decrease during differentiation into NPCs. In contrast, 27 chaperome components were increased during neural differentiation. Among the 44 chaperome components decreased in terminally differentiated neurons compared with hESCs, 28 proteins were already down-regulated during differentiation into NPCs.

Notably, several subunits of the chaperonin TRiC/CCT complex decreased at both transcript and protein levels during neural differentiation of hESCs. TRiC/CCT is required for cell viability as a key component of the proteostasis network that facilitates the folding of approximately 10% of the eukaryotic proteome (Yam, Xia et al. 2008, Lopez, Dalton et al. 2015). TRiC/CCT not only assists the folding of newly synthesized proteins (Etchells, Meyer et al. 2005) but also binds to misfolded proteins regulating their aggregation (Priya, Sharma et al. 2013). Given the crucial role of this molecular chaperonin in proteostasis, we further assessed its regulation on pluripotent stem cells. In eukaryotes, the hetero-oligomeric TRiC/CCT complex consists of two stacked rings of eight paralogous subunits each (Leitner, Joachimiak et al. 2012). In our quantitative proteomics assay, we found that CCT3, CCT4 and CCT8 subunits are significantly increased in hESC compared with NPCs. We confirmed these results by western blot analysis and found that other subunits also decrease during neural and neuronal differentiation (*i.e.*, CCT2, CCT6A and CCT7) (Figure 5a). The decrease in the protein amount of CCT subunits correlated with a reduction of the mRNA levels during differentiation (Figure 5b). Differentiation into either endoderm or mesoderm induced a similar decrease (Figure 5c-f).

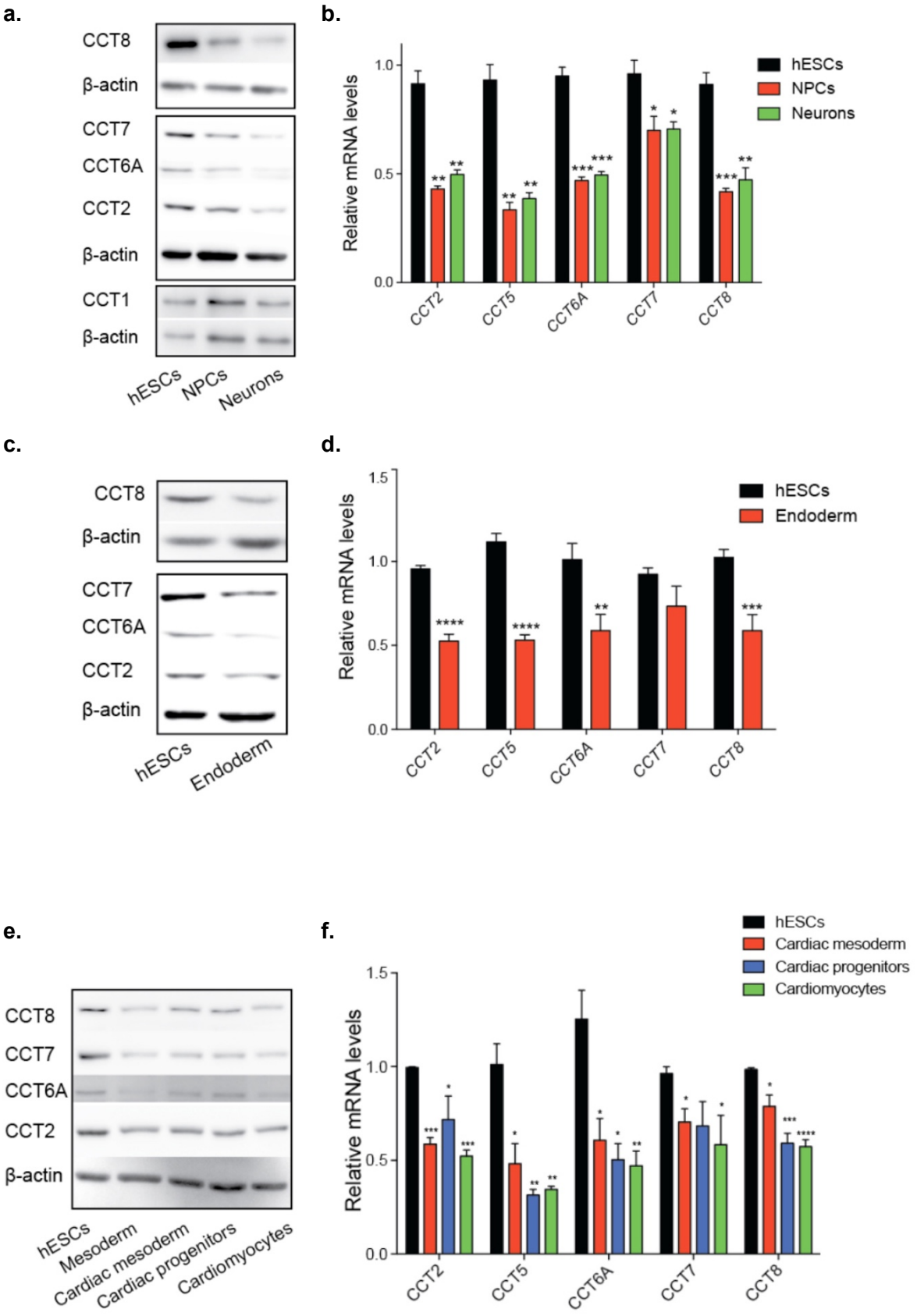

Figure 5. Expression of CCT subunits decreases during differentiation. (a), **(c)** and **(e)** Western blot analysis with antibodies to CCT8, CCT7, CCT6A, CCT2 and CCT1. β-actin is the loading control. In **(a)** and **(c)** the differentiation was performed with the H9 hESC line whereas in **(e)** H1 hESCs were used. **(b)** CCT subunits relative expression to H9 hESCs represents the mean ± s.e.m. of three independent experiments. **(d)** CCT subunits relative expression to H9 hESCs represents the mean ± s.e.m. (n= 7 independent experiments). **(f)** CCT subunits relative expression to H1 hESCs represents the mean ± s.e.m. of three independent experiments. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001).

Consistent with the ability of hESCs to self-renew indefinitely while maintaining their undifferentiated state (Thomson, Itskovitz-Eldor et al. 1998, Miura, Mattson et al. 2004), the expression of CCT subunits and pluripotency markers did not differ depending on the passage number (Figure 6a-b). Taken together, our results indicate that hESCs are able to maintain enhanced expression of CCT subunits under unlimited proliferation in their undifferentiated state. However, the levels of subunits such as CCT8 or CCT2 decrease when hESCs differentiate into distinct cell lineages. Thus, increased levels of CCT subunits could be an intrinsic characteristic of hESCs linked to their immortality and identity.

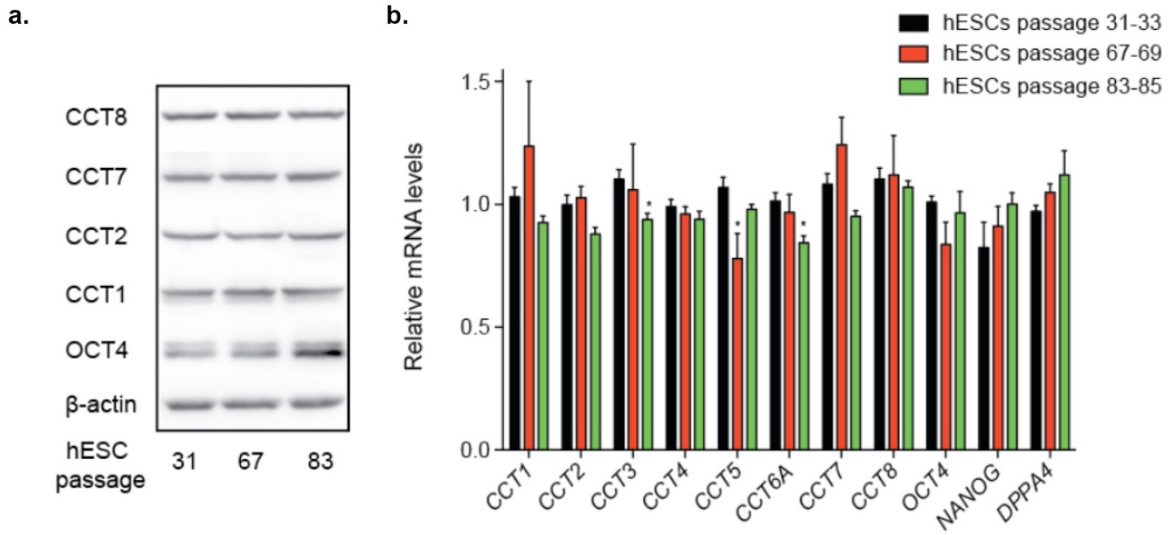

Figure 6. H1 hESCs and iPSCs exhibit increased expression of CCT subunits. (a) Protein levels of CCT subunits do not differ with passage in H9 hESCs. **(b)** Data represent the mean ± s.e.m. of the relative expression levels to H9 hESCs passage 31-33 (n= 3 independent experiments). All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001).

3.3.1.2 Increased expression of CCT8 induces TRiC/CCT assembly

Prompted by these findings, we asked whether increased levels of CCT subunits resulted into more assembled TRiC/CCT complexes in hESCs. Although both hESCs and NPCs have similar levels of total CCT1 subunits (Figure 6a, Figure 7a-b**)** hESCs lines exhibited a dramatic increase in the assembly of TRiC/CCT in the form of two stacked rings (Figure 7a-b). Similarly, iPSCs also had increased TRiC/CCT assembly compared with differentiated cells (Figure 7c). Since all the subunits are required for TRiC/CCT function (Spiess, Meyer et al. 2004), downregulated CCT subunits could become structural limiting factors during differentiation and modulate the decrease of TRiC/CCT assembly. An intriguing possibility is that specific subunits can also function as assembly activators. A

comparison between the levels (relative to CCT1) of the different subunits in hESCs and NPCs showed that CCT8 is the most abundant subunit in both cell types despite decreasing during differentiation (Figure 7d).

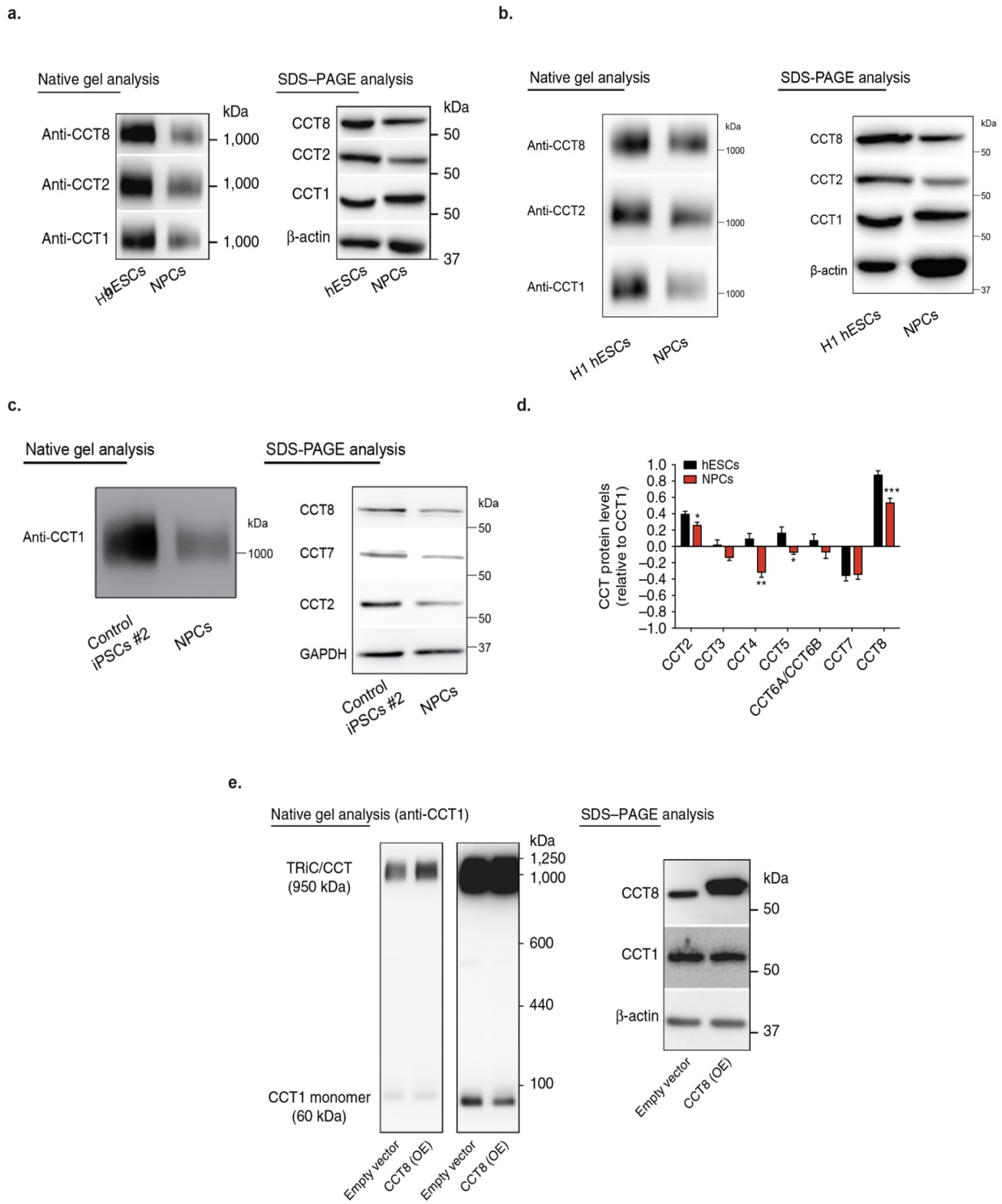

Figure 7. Ectopic expression of CCT8 is sufficient to increase TRiC/CCT assembly. (a-c) Native gel electrophoresis of H9, H1, iPSC and NPCs extracts. Extracts were resolved by SDS-PAGE and immunoblotting for analysis of total CCT subunit levels and β-actin loading control. **(d).** Label-free quantification (LFQ) of CCT protein levels relative to CCT1. All detected CCT subunits were quantified by their log2 fold change in LFQ intensities relative to CCT1. Graphs represent the mean ± s.e.m. (hESCs (n= 9), NPCs (n= 6)). **(e).** Native gel electrophoresis of HEK293T cell extracts followed by immunoblotting with CCT1 antibody (two different exposure times of the same membrane are shown). Extracts were resolved by SDS-PAGE and immunoblotting for analysis of total CCT8 and CCT1 subunit levels and β-actin loading control.

These findings indicate that CCT8 is not stoichiometric limiting for TRiC/CCT assembly. Thus, we asked whether an increase in the total protein levels of CCT8 could trigger TRiC/CCT assembly. Strikingly, ectopic expression of CCT8 induced an increase in TRiC/CCT assembly whereas the total protein levels of CCT1 remained similar (Figure 7e). In contrast, we found a decrease in the levels of CCT1 in its monomeric form upon CCT8 overexpression (Figure 7e). Collectively, our results suggest that hESCs have an intrinsic proteostasis network characterized by high levels of TRiC/CCT complex. During differentiation, the levels of distinct CCT subunits decrease and diminish the assembly of TRiC/CCT chaperonin. In addition, we identified CCT8 as a potential activator of TRiC/CCT assembly by using hESCs as a model to study proteostasis.

3.3.1.3 Loss of CCT subunits affects hESC identity

With the strong connection between hESC identity, CCT8 expression and enhanced assembly of TRiC/CCT complex, we asked whether increased levels of CCT8 contribute to maintain hESC function. Given that TRiC/CCT is essential for cell viability (Lopez, Dalton et al. 2015), loss of CCT subunits induces cell death and detachment (Figure 8a). To avoid these effects, we induced a mild knockdown of approximately 30-50% by transient lentiviral transduction. Notably, downregulation of CCT8 resulted in decreased levels of pluripotency markers (Figure 8b). We hypothesized that high CCT8 expression impinges upon hESC function via enhanced assembly of TRiC/CCT complex. Since mutation or loss of a single subunit is sufficient to impair the activity of the complex (Spiess, Meyer et al. 2004), we knocked down other subunits (*i.e.*, *CCT2*, *CCT6A* and *CCT7*) to determine whether increased TRiC/CCT is required for hESC function. As with CCT8 knockdown, decreased levels of these subunits affected the expression of pluripotency markers (Figure 8b). We performed these experiments in an independent hESC line as well as iPSC lines and obtained similar results (Figure 8c-d).

In addition, loss of CCT levels induced the expression of markers of the distinct germ layers (Figure 9b). Since we observed an up-regulation in specific markers of the three germ layers, our data suggest that hESCs undergo a decline of pluripotency upon knockdown of CCT subunits but they do not differentiate into a particular cell lineage. Although we cannot discard a role of free monomeric CCT subunits, our results indicate that increased levels of the TRiC/CCT complex are required for hESC identity.

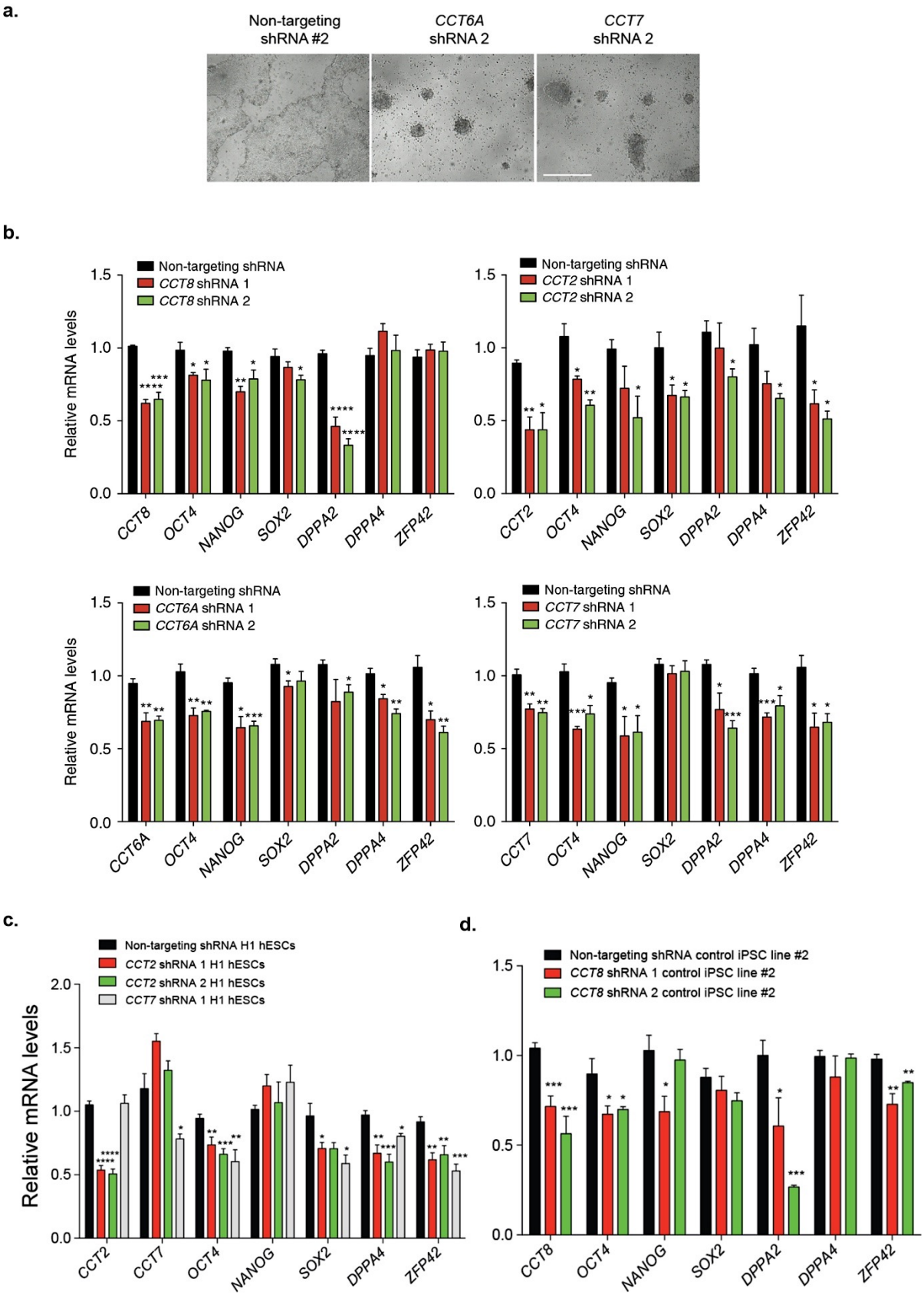

Figure 8. Knockdown of CCT subunits affects pluripotency of PSCs. (a) Acute knockdown of CCT subunits induces cell death and detachment of H9 hESCs**.** Representative images of two independent experiments. **(b-d)** Real Time PCR analysis of pluripotency markers in H9, H1 and iPSCs. Knockdown of CCT8, CCT2, CCT6A or CCT7 decrease the expression of pluripotency markers. Graphs (relative expression to NT shRNA) represent the mean ± s.e.m. of at least three independent replicates. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001), **** (P<0.0001).

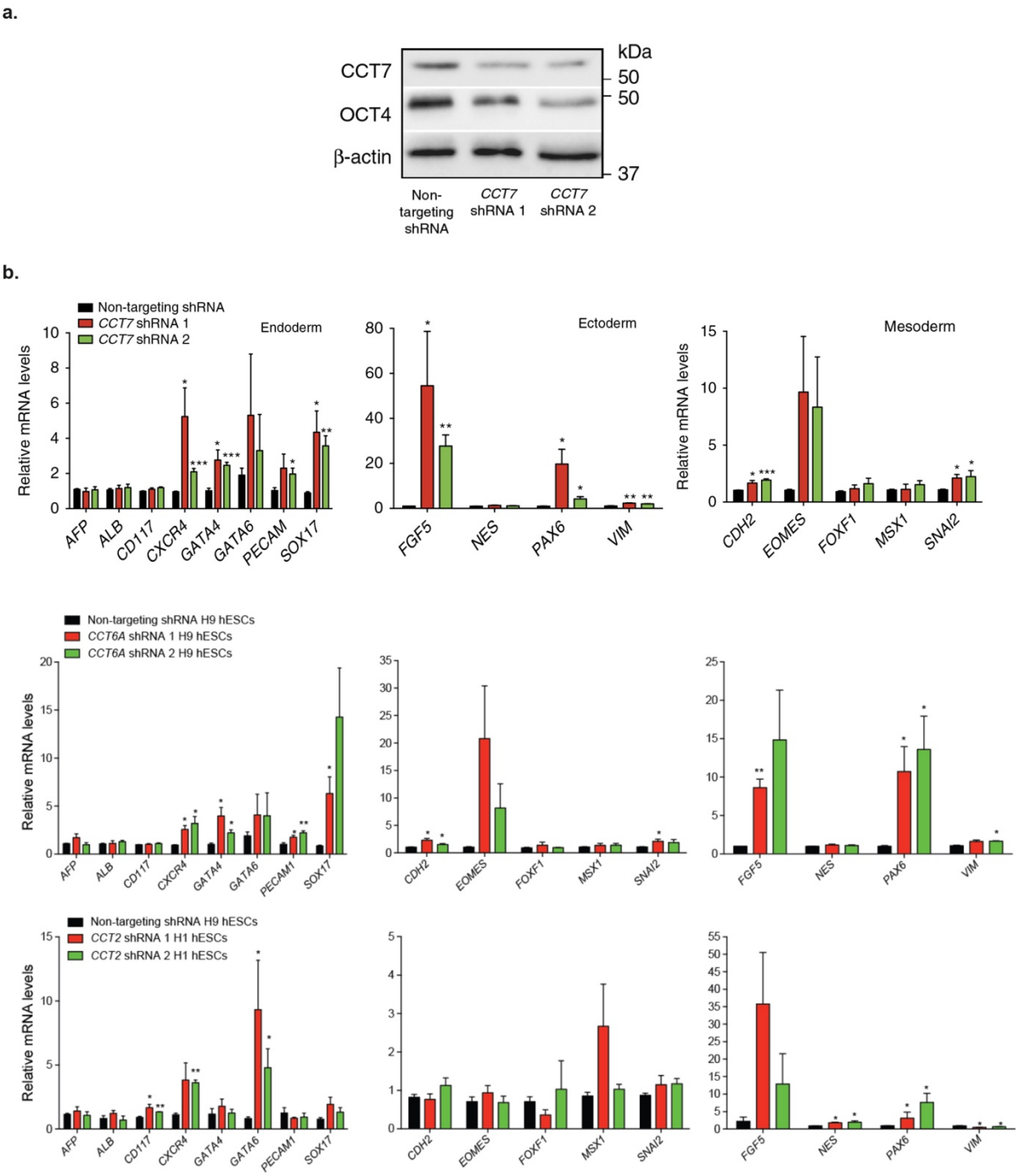

Figure 9. Knockdown of *CCT* subunits affects the expression of germ layer markers in different cell lines. ***(a)*** Knockdown of *CCT7* induces a decrease in OCT4 protein levels. β-actin is the loading control. **(b)** Real Time PCR analysis of germ layer markers in H9 , H1 and iPSCs (relative expression to NT shRNA). Data represent Graph represents the mean ± s.e.m. of four independent experiments. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), *** (P<0.001).

3.3.1.4 TRiC/CCT complex determines proteostasis of pluripotent stem cells

Because the TRiC/CCT complex modulates aggregation of damaged and misfolded proteins (Priya, Sharma et al. 2013), we asked whether this chaperonin is required for increased proteostasis of pluripotent stem cells. To tackle this question, we used iPSCs derived from Huntington's disease patients (HD-iPSCs). HD is an autosomal dominant neurodegenerative disorder caused by the expansion of a CAG triplet repeat region in the *huntingtin* gene (*HTT*), which translates into a polyglutamine stretch (polyQ) in the protein and results in proteotoxicity and aggregation (Finkbeiner 2011). In its wild-type form, HTT contains 6-35 glutamine residues. However, in individuals affected by HD, it contains greater than 35 glutamine residues and longer repeats predict younger disease onset (Langbehn, Hayden et al. 2010, Finkbeiner 2011). Here we used three different control iPSC lines and HD-iPSCs from four donors with different allelic series (*i.e.*, polyQ57, polyQ60, polyQ71 and polyQ180). These HD-iPSC lines possess one mutant copy of huntingtin gene but also one normal allele (Figure 10a-b). Although the levels of mutant HTT were lower compared to the normal protein, HD-iPSCs exhibited significant amounts of mutant polyQ-expanded HTT protein (Figure 10a-b). In iPSCs that express longer CAG repeat expansions (polyQ180), the differences between the levels of mutant and normal HTT were more dramatic (Figure 10b). Nevertheless, we confirmed the expression of mutant HTT in these iPSCs by using an antibody that detects remarkably better polyQ-expanded HTT than wild type HTT (Trottier, Lutz et al. 1995, Consortium 2012) (Figure 10b-e). To examine the aggregation of mutant HTT in HD-iPSCs, we performed filter trap analysis that allows for quantification of polyQ aggregates (Vilchez, Morantte et al. 2012). Although HD-iPSCs expressed significant levels of polyQ-expanded HTT, these cells did not exhibit accumulation of detectable polyQ aggregates compared with control iPSCs as assessed by both filter trap and immunohistochemistry analyses (Figure c, e, f and g). However, a collapse in proteostasis induced by proteasome inhibition triggered the accumulation of polyQ aggregates in HD-iPSCs (Jeon, Lee et al. 2012) (Figure 10f). Loss of CCT subunits enhances aggregation of mutant HTT and wor6sens HD-related changes in yeast, *C. elegans* and mammalian neuronal models (Nollen, Garcia et al. 2004, Kitamura, Kubota et al. 2006, Tam, Geller et al. 2006). Thus, increased TRiC/CCT assembly induced by high levels of CCT subunits could contribute to maintain the proteostasis of mutant HTT in iPSCs. We found that knockdown of CCT8 and other CCT subunits trigger the accumulation of polyQ aggregates without affecting the mutant HTT total protein levels in HD-iPSCs (Figure 10d-e).

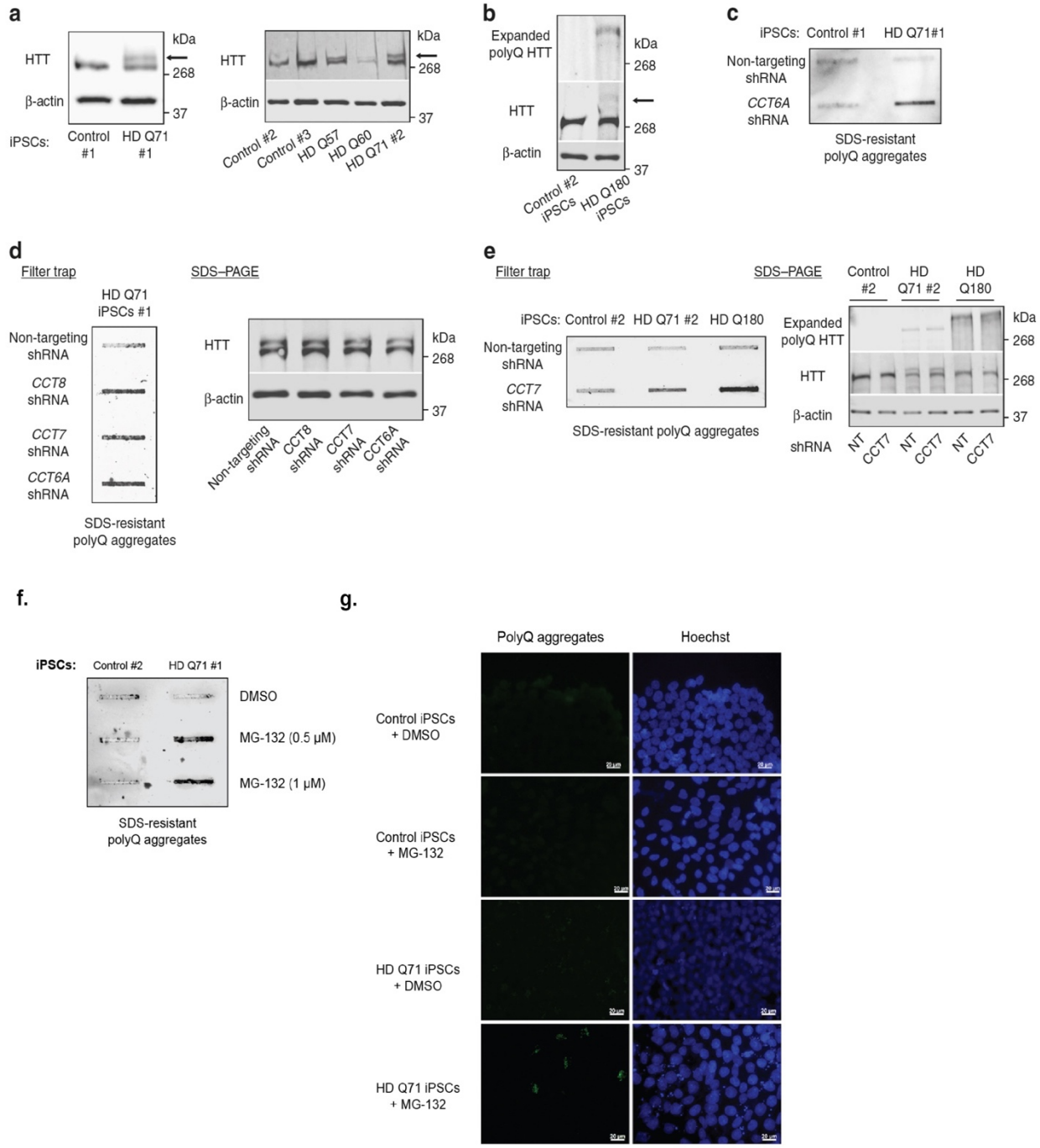

Figure 10. Knockdown of CCT subunits impairs proteostasis of pluripotent stem cells. (**a**) Western blot analysis of control and HD-iPSC lysates with antibodies to HTT and β-actin. Arrow indicates mutant polyQ-expanded HTT. (**b**) In HD polyQ180-expressing iPSCs, the levels of mutant HTT are dramatically decreased compared with normal HTT copy. The expression of polyQ180 HTT was confirmed by using an antibody that detects polyQ-expanded mutant HTT. (**c**) Filter trap analysis shows that HD-iPSCs does not have increased levels of polyQ aggregates compared to control iPSCs (detected by anti-polyQ-expansion diseases marker antibody). However, knockdown of a single CCT subunit triggers the accumulation of polyQ aggregates. (**d**) Knockdown of different CCT subunits in HD polyQ71 iPSC line #1 results in a similar increase of polyQ aggregates. Right panel: SDS-PAGE analysis with antibodies to HTT and β-actin loading control. (**e**) Knockdown of a single CCT subunit triggers the accumulation of polyQ aggregates in both polyQ71 iPSC line #2 and polyQ180 iPSCs. Right panel: PolyQ-expanded mutant HTT antibody was used to confirm that the total protein levels of mutant HTT do not change upon knockdown of CCT subunits. (**f**) Filter trap analysis shows that proteasome inhibition with MG-132 for 12 h results in increased levels of polyQ aggregates in the pellet of HD Q71-iPSCs (line #1). However, proteasome inhibition does not induce accumulation of polyQ aggregates in control iPSC line #2 (polyQ21). (**g**) Immunocytochemistry of control iPSC line #2 (Q21) and HD Q71-iPSC line #1 treated with 5 μM MG-132 (proteasome inhibitor) for 12 h. Expanded polyQ antibody was used to detect polyQ aggregates. Cell nuclei were stained with Hoechst 33342. Scale bar represents 20 μm.

These results were observed in all the HD-iPSC lines tested whereas knockdown of CCT subunits did not induce accumulation of polyQ aggregates in control iPSCs (Figure 10c-e). Remarkably, loss of different CCT subunits (i.e., CCT2, CCT6A, CCT7 and CCT8) had similar effects (Figure 10d), indicating that the TRiC/CCT complex modulates polyQ aggregation in HD-iPSCs. HD-iPSCs represent a valuable resource to gain mechanistic insights into HD (Consortium 2012). Neuronal dysfunction and death occurs in many brain regions in HD, but striatal neurons expressing cAMP-regulated phosphoprotein (DARPP32) undergo the greatest neurodegeneration (Vonsattel, Keller et al. 2008). HD-iPSCs can be terminally differentiated into neurons (including DARPP32-positive cells) that exhibit HD-associated phenotypes such as cumulative risk of death over time and increased vulnerability to excitotoxic stressors (Consortium 2012). Furthermore, proteotoxic stress via autophagy inhibition or oxidative stress results in higher neurodegeneration of HD cells compared with controls (Consortium 2012). Despite these phenotypes, mutant HTT-expressing neurons present important limitations for disease modeling such as lack of robust neurodegeneration, polyQ aggregates and gene expression changes resembling HD (Consortium 2012, Jeon, Lee et al. 2012). Despite the efforts to detect polyQ aggregates under different conditions (for instance adding cellular stressors), the presence of inclusions has not been observed in neurons derived from HD-iPSCs (Consortium 2012, Jeon, Lee et al. 2012). The lack of inclusions in these cells could reflect the long period of time before aggregates accumulate in HD (Consortium 2012). Consistently, HD human neurons do not accumulate detectable polyQ aggregates at 12 weeks after transplantation into HD rat models whereas these inclusions are observed after 33 weeks of transplantation (Jeon, Lee et al. 2012). The lack of inclusions in HD neurons could reflect the long period of time before aggregates accumulate in HD (Consortium 2012). To assess whether loss of CCT subunits triggers neurodegeneration and polyQ aggregation in these cells, we differentiated three HD-iPSC lines (Q57, Q71 and Q180) into striatal neurons (Aubry, Bugi et al. 2008). Among those cells expressing the neuronal marker MAP2, approximately 50% also expressed DARPP32 as previously described (Aubry, Bugi et al. 2008, Consortium 2012) (Figure 11a). As with proteasome inhibitor treatment, we could not detect polyQ aggregates in mutant HTT-expressing neurons upon knockdown of CCT subunits by either immunofluorescence or filter trap (Figure 11b, c). However, knockdown of CCTs resulted in increased cell death of HD neuronal cultures assessed by cleaved caspase-3 quantification whereas it did not induce cell death in control neurons (Figure 11d). In contrast, proteasome inhibition triggered cell death at the same extent in both control and HD neurons (Consortium 2012) (Figure 11d).

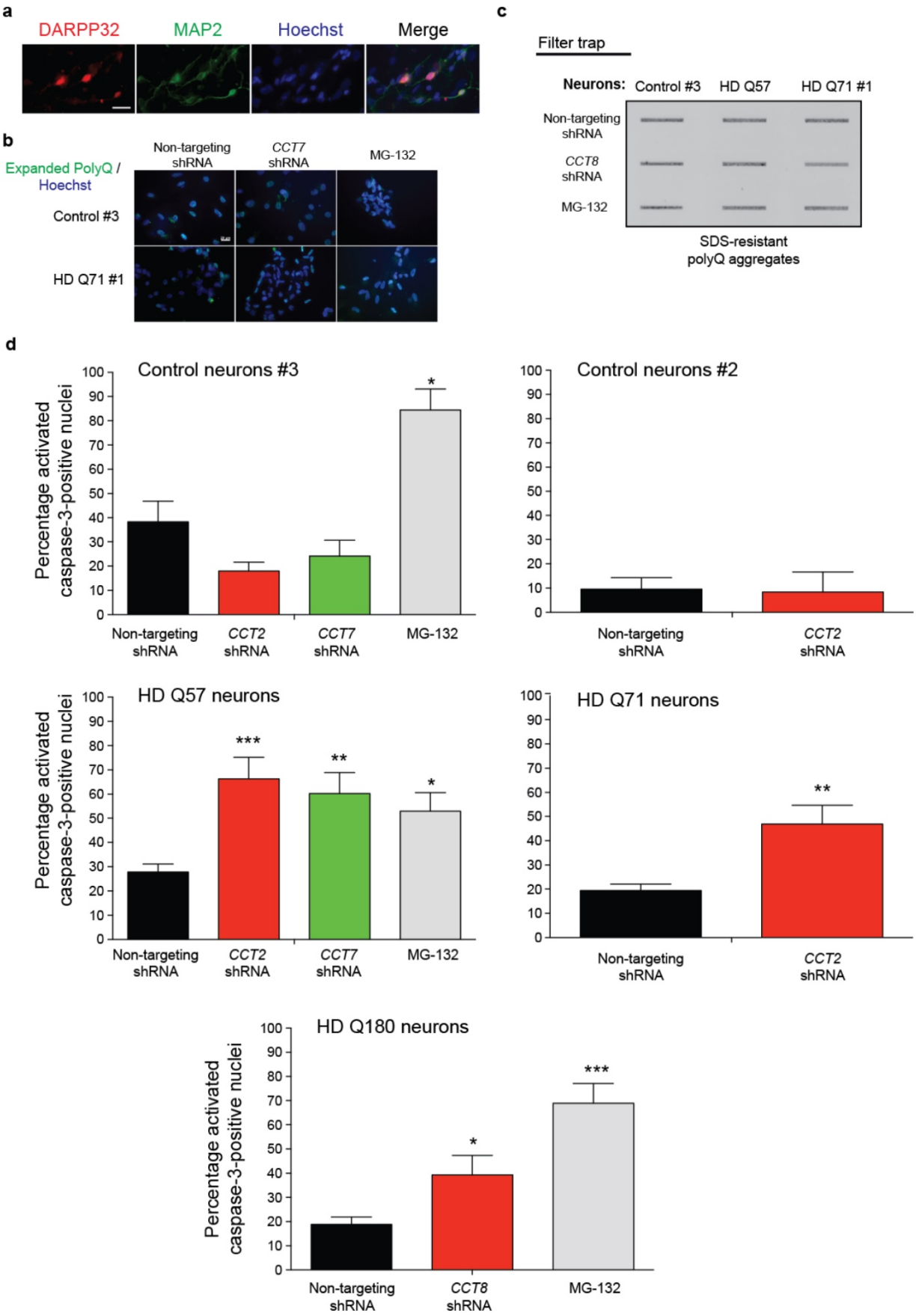

Figure 11. Dysfunction of TRiC/CCT induces activation of caspase-3 in neurons derived from HD-iPSCs. **(a)** Immunofluorescence of HD Q57 neurons. DARPP32 staining was used as a marker of striatal neurons. MAP2 and Hoechst 33342 staining were used as markers of neurons and nuclei, respectively. Scale bar represents 50 µm. Among MAP2-positive neurons, approximately 50% were also DARPP32-positive. Images are representative of two independent experiments. We obtained similar efficiency of differentiation with other lines (i.e., Control #2, Control #3, HD Q71 line #2 and HD Q180).

(b) Immunofluorescence of neurons derived from iPSCs with expanded polyQ antibody and Hoechst 33342. Scale bar represents 20 μm. The images are representative of three independent experiments. **(c)** Filter trap analysis of neurons derived from iPSCs. Expanded polyQ antibody was used to detect polyQ aggregates. MG-132: 5μM MG-132 for 24 h. The images are representative of two biological replicates. **(d)** Percentage of activated caspase-3- positive nuclei. Graph represents the mean ± s.e.m. of the percentage observed in 4 independent neuronal differentiation experiments (we assessed approximately 150 total nuclei in each independent experiment). MG-132: 5 μM MG-132 for 24 h. Activated caspase-3-positive nuclei were stained with antibody to cleaved caspase-3. Total number of cells was evidenced after staining of nuclei with Hoechst 33342. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *($P<0.05$), **($P<0.01$), *** ($P<0.001$).

These results indicate that mutant HTT-expressing neurons are more susceptible to TRiC/CCT dysfunction and provide a link between downregulation of CCTs with onset of neurodegeneration during aging in HD.

3.3.1.5 Somatic overexpression of CCT8 extends organismal lifespan

Our results indicate that increased TRiC/CCT complex is a key determinant of proteostasis of immortal hESCs/iPSCs. However, the levels of CCT subunits decreased upon differentiation. During human brain aging, the expression of CCTs is further repressed (Brehme, Voisine et al. 2014). With the strong correlation between *cct* levels, differentiation and aging, we asked whether inducing TRiC/CCT assembly in somatic post-mitotic cells could have a positive role in longevity. To examine the impact of TRiC/CCT on organismal aging and proteotoxic resistance, we used the nematode *C. elegans*. In this organism, CCT transcripts are detected in most tissues and developmental stages (Hill, Hunter et al. 2000). The role of TRiC/CCT complex in proliferating cells during *C. elegans* development has been extensively studied (Lundin, Srayko et al. 2008). Disruption of TRiC/CCT assembly by knockdown of different CCT subunits causes a variety of defects in cell division and results in embryonic lethality (Gonczy, Echeverri et al. 2000, Simmer, Moorman et al. 2003, Lundin, Srayko et al. 2008, Green, Kao et al. 2011). These effects are partially mediated by a collapse in microtubule function as a consequence of diminished folding of tubulin by TRiC/CCT (Lundin, Srayko et al. 2008). In addition, loss of different *cct* subunits during post-embryonic developmental stages results in larval arrest, body morphology alterations as well as defects in developing gonads and sterility, indicating a key role of the TRiC/CCT complex in *C. elegans* development (Lundin, Srayko et al. 2008). In adult worms, the only proliferating cells are found in the germline whereas somatic tissues are formed exclusively by post-mitotic cells (Hubbard 2007). To examine the expression of CCTs in germ cells, we dissected the germline and intestine from adult *C. elegans* and compared the levels of CCT subunits by immunohistochemistry. We found that CCT-1 subunit is enhanced in germ cells compared with the intestine (Figure 12a), suggesting that CCTs are highly expressed in the germline. Notably, knockdown of *cct* subunits during adulthood dramatically decreased the number of germ cells destabilizing the germline (Figure 12b). Accordingly, we observed a dramatic decrease in the number of laid eggs after 2 days of *cct* RNAi treatment during adulthood (Figure 12c). Overall, these data suggest that high levels of TRiC/CCT complex are essential for proliferating cells and germline stability. However, knockdown of *cct* subunits during adulthood did not decrease lifespan in wild-type worms (Figure 12d).

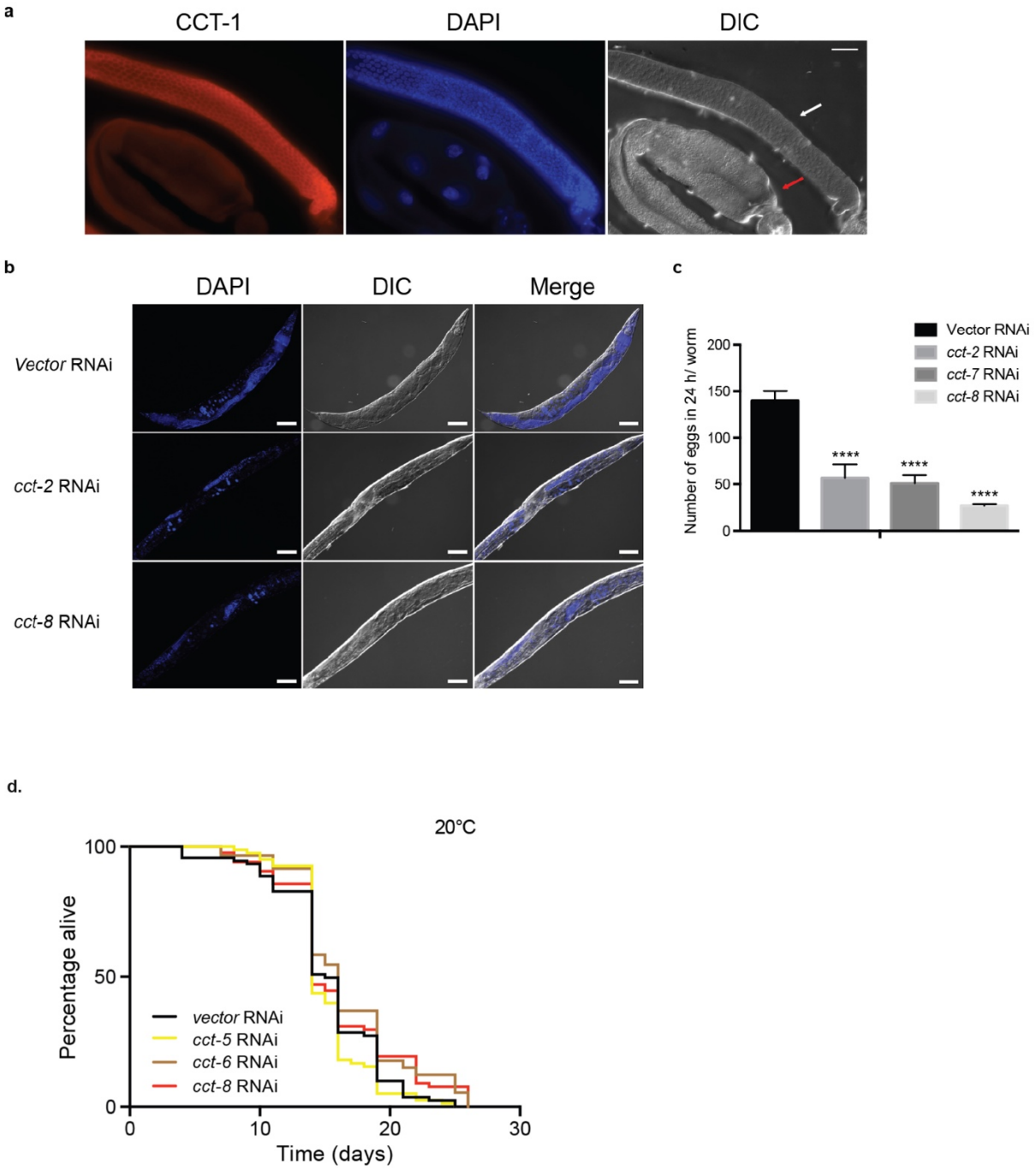

Figure 12 Knockdown of CCT subunits destabilizes *C. elegans* germline. (a) Gonad and intestine immunostaining with CCT-1 antibody from day 1-adult wild-type worms. Cell nuclei were stained with DAPI. White arrow indicates gonad and red arrow indicates intestine. Scale bar represents 20 µm. DIC= differential interference contrast. The images are representative of two independent experiments. **(b)** Nuclei staining with DAPI of day 4 adult wild-type worms. Knockdown of cct subunits during adulthood reduces the number of germ line cells. RNAi was initiated at day 1 of adulthood. Scale bar represents 100 µm. The images are representative of two independent experiments. **(c)** Number of eggs laid per worm during 24 h. RNAi was initiated at day 1 of adulthood. We started counting the number of laid eggs after 1 day of RNAi treatment. Graph represents the mean ± s.e.m. of 5 independent experiments (in each independent experiment, we counted and averaged the number of eggs laid by 6 worms for each treatment). **(d)** Loss of cct subunits during adulthood does not significantly decrease lifespan of wild-type worms at 20°C. All the statistical comparisons were made using the log-rank (Mantel–Cox) method. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: **** (P<0.0001).

To assess the expression of CCT subunits in somatic tissues of adult *C. elegans*, we generated a GFP transcriptional reporter construct for *cct-8* gene. Although we did not observe GFP expression in germ cells as a result of germline silencing of transgenes (Kelly, Xu et al. 1997, McKay, Johnsen et al. 2003), these experiments confirmed wide expression of *cct-8* in somatic cells including neurons or body wall muscle cells (Figure 13 a-b). Somatic expression pattern of *cct-8* resembled other *cct* subunits (*e.g.*, *cct-1*, *cct-2* and *cct-7*) showing a high expression in pharynx and tail (McKay, Johnsen et al. 2003, Lundin, Srayko et al. 2008) (Figure 13a-b).

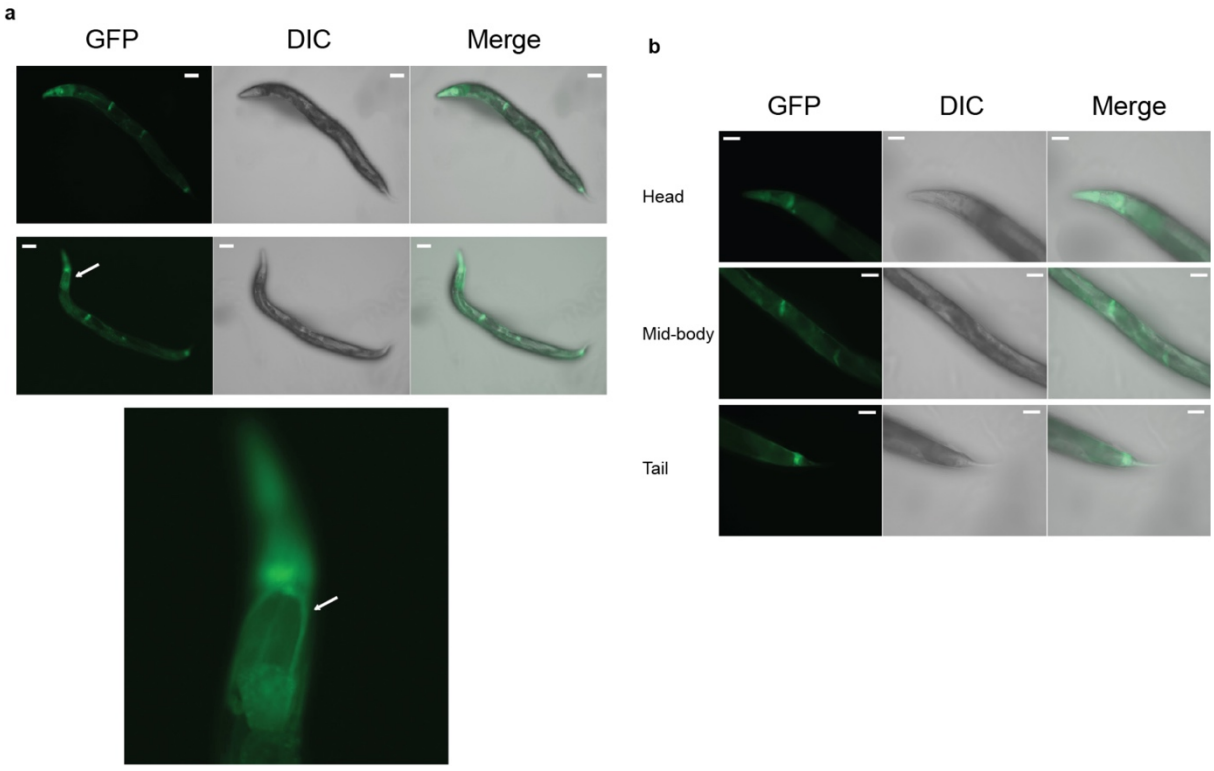

Figure 13. *cct-8* is widely expressed in somatic tissues. (a) Representative images of GFP expressed under control of the cct-8 promoter in whole adult worm. Scale bar represents 100 µm. Arrow indicates neurons. **(b)** Adult expression of GFP under cct-8 promoter in the head, mid-body and tail regions. Scale bar = 50 µm. The images are representative of three independent experiments.

In aging organisms, post-mitotic somatic tissues undergo a gradual deterioration and become particularly susceptible to age-associated protein aggregation diseases. Thus, we asked whether increasing the levels of the TRiC/CCT complex in somatic tissues could delay the aging of post-reproductive organisms. To examine this hypothesis, we overexpressed CCT8, a subunit that promotes TRiC/CCT assembly in mammalian cells (Figure 14e). For this purpose, we induced ectopic expression of *cct*-8 under *sur-5* promoter, which is

expressed ubiquitously in somatic tissues but not in the germline (Gu, Orita et al. 1998, Vilchez, Morantte et al. 2012, Vilchez, Morantte et al. 2012). Notably, somatic overexpression (OE) of *cct-8* was sufficient to extend the lifespan of *C. elegans* under normal conditions (20°C) (Figure 14b). These worms exhibited up to 20% increased median lifespan compared with the control strain and other *cct*-overexpressing worms (Figure 14b). Since overexpression of a single *cct* subunit did not change the levels of other subunits, the longevity phenotype can be attributed specifically to *cct-8* (Figure 14a). Interestingly, the lifespan extension induced by ectopic expression of *cct-8* was more dramatic at 25°C (Figure 14c). Under this temperature, *cct-8(OE) C. elegans* lived up to 40% longer than the control strain. Overexpression of *cct-2* also increased lifespan significantly at 25°C, although to a lesser extent than *cct-8* (Figure 14c). Since heat stress challenges the structure of proteins and triggers the accumulation of misfolded proteins, our results indicate that both *cct-8* and *cct-2* extend longevity by sustaining the integrity of the proteome during adulthood.

To examine whether these pro-longevity effects are induced through modulation of the TRiC/CCT complex, we knocked down the expression of a different subunit. Given that *cct* subunits are required during larval development, we initiated RNAi treatment during adulthood. Interestingly, knockdown of *cct-6* partially reduces the longevity phenotype of both *cct-8(OE)* and *cct-2(OE)* worms whereas it did not affect the lifespan of the control strain (Figure 14d). By native gel electrophoresis analysis, we confirmed that overexpression of *cct-8* in somatic tissues increases TRiC/CCT assembly in the form of two stacked rings (Figure 14e). Collectively, these results suggest that ubiquitous somatic overexpression of *cct-8* induces longevity via modulation of TRiC/CCT assembly, particularly, under proteotoxic conditions.

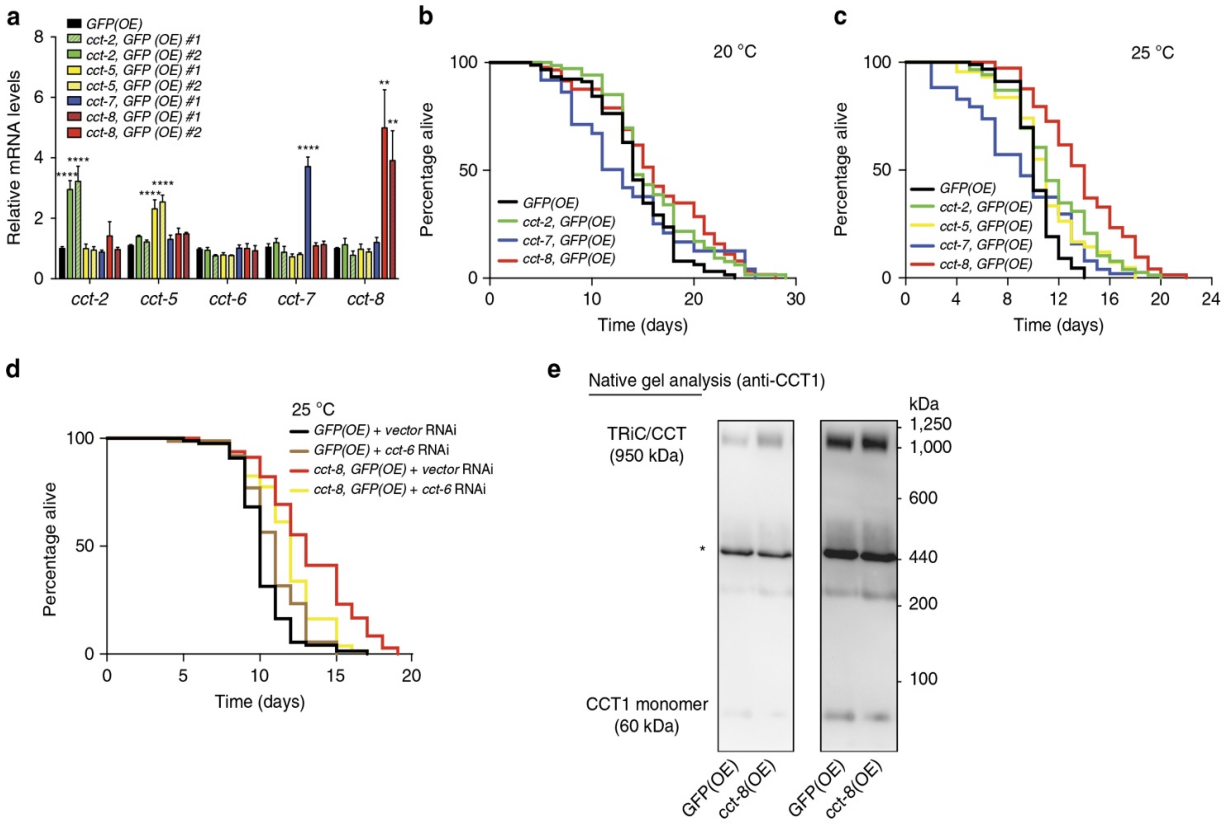

Figure 14 Somatic increased expression of cct-8 induces TRiC/CCT assembly and extends longevity. (a) Data represent the mean ± s.e.m. of the relative expression levels to *GFP(OE)* worms (n= 4 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. P-value: **(P<0.01), **** (P<0.001). (**b**), (**c**) and (**d**) Each lifespan graph shows a Kaplan-Meier survival plot of a single representative experiment. In each graph, experimental and control animals were grown in parallel. n= total number of uncensored animals/total number (uncensored + censored) of animals observed in each experiment. P-values refer to experimental and control animals in a single lifespan experiment. (**b**) *cct-8(OE)* extends lifespan at 20°C (log rank, P<0.0001). *GFP(OE)*: median= 14, n= 73/96; *cct-2, GFP(OE)*: median= 14, n= 65/96; *cct-7, GFP(OE)*: median= 13, n= 46/74; *cct-8, GFP(OE)*: median= 16, n= 70/96. (**c**) Both *cct-8(OE)* and *cct-2(OE)* worms live longer compared to control *GFP(OE)* strain at 25°C (log rank, P<0.0001). *cct-8(OE)* worms live longer compared with *cct-2(OE)* and other *cct(OE)* strains (log rank, P<0.0001). *GFP(OE)*: median= 10, n= 89/96; *cct-2, GFP(OE)*: median= 11, n= 79/96; *cct-5, GFP(OE)*: median= 11, n= 42/50; *cct-7, GFP(OE)*: median= 9, n= 56/77; *cct-8, GFP(OE)*: median= 14, n= 82/96. (**d**) Knockdown of *cct-6* reduces the long lifespan induced by *cct-8(OE)* (log rank, P= 0.0001). In contrast, loss of *cct-6* does not decrease the lifespan of *GFP(OE)* worms. RNAi was initiated at day 1 of adulthood. *GFP(OE)* fed *empty vector* RNAi bacteria: median= 10, n= 74/96; *GFP(OE)* fed *cct-6* RNAi bacteria: median= 11, n= 73/96; *cct-8, GFP(OE)* fed *empty vector* bacteria: median= 13, n= 77/96; *cct-8, GFP(OE)* fed *cct-6* RNAi bacteria: median= 12, n= 79/96. (**e**) Native gel electrophoresis of *GFP(OE)* and *cct-8,GFP(OE)* worm extracts followed by immunoblotting with CCT-1 antibody (two different exposure times of the same membrane are shown). Overexpression of cct-8 induces an increase in the assembly of TRiC/CCT in the form of two stacked rings. (*) indicates a complex of approximately 475 kDa which is strongly detected in worm lysates. The molecular weight suggests that this signal could correspond to single ring TRiC/CCT assembled forms.

3.3.1.6 *CCT-8* determines proteotoxic stress resistance

During aging, organisms lose their ability to maintain proteostasis and respond to proteotoxic stress (Taylor and Dillin 2011, Lopez-Otin, Blasco et al. 2013). With age, the expression of several *cct* subunits significantly decreased in *C. elegans* whereas the levels of *cct-1* remained similar (Figure 15a-b). At day 5 of adulthood, several *cct* subunits (*e.g.*, *cct-2*, *cct-5* and *cct-8*) were already downregulated (Figure 15a-b).

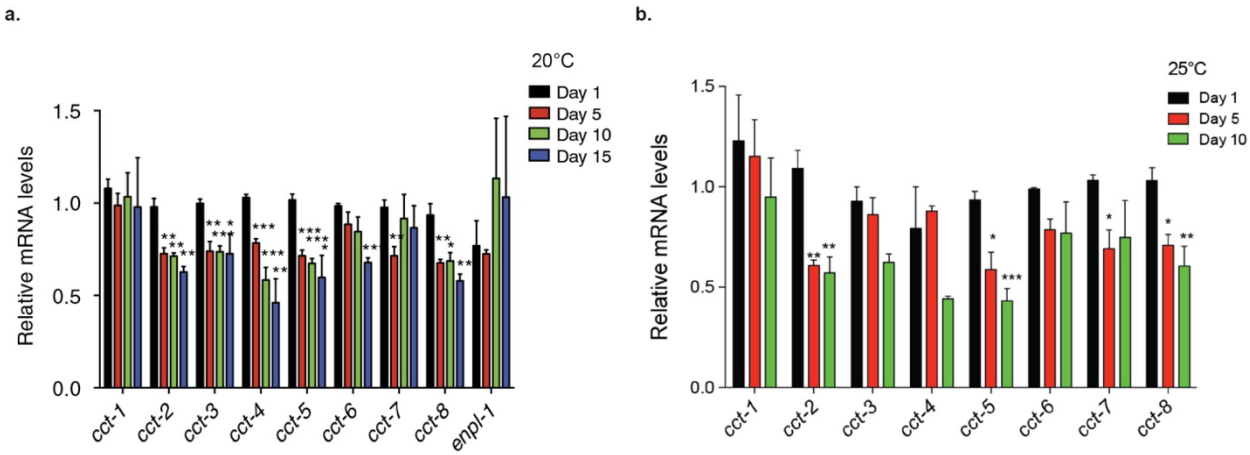

Figure 15 Expression of specific *cct* subunits decrease with age. (a) The expression of several cct subunits decreases with age. In contrast, we did not find significant differences in cct-1 and enpl-1, the *C. elegans* orthologue to the human chaperone HSP90B1. Data represent the mean ± s.e.m. of the relative expression levels to day 1 adult worms grown at 20°C or 25°C **(b)** These experiments were performed with the sterile control strain fer-15(b26)II;fem-1(hc17) (n= 4 independent experiments).

To examine whether somatic *cct-8(OE)* ameliorates the age-associated demise in proteotoxic stress responses, we induced acute heat stress (34°C) at different ages (*i.e.*, day 1 and day 5). Severe heat stress dramatically affects survival and activates the heat-shock response (HSR), an essential mechanism to ensure proper cytosolic protein folding and alleviate proteotoxic stress (Vabulas, Raychaudhuri et al. 2010). Under heat stress (34°C), *cct-8(OE)* worms did not survive significantly longer compared with control strain at day 1 of adulthood (Figure 16a). However, *cct-8(OE)* worms were markedly more resistant to proteotoxicity than control strains when subjected to heat stress at day 5 of adulthood (Figure 16b). Although to a lesser extent, *cct-2(OE)* also conferred resistance to heat stress (Figure 16b). As a more formal test, we asked whether animals with reduced HSR had increased survival when *cct-8* was overexpressed (Figure 16b). Heat-shock transcription factor (HSF-1) activates the HSR and is required for proteotoxicity resistance and adult lifespan (Morimoto 2011). Thus, we induced downregulation of HSR via silencing *hsf-1* expression. Notably, *hsf-1*-RNAi-treated *cct-8(OE)* worms lived markedly longer compared to control worms under

the same treatment (Figure 16c). These results indicate that *cct-8(OE) C. elegans* can significantly overcome the loss of a key transcription factor such as *hsf-1*, which is not only required for HSR but also lifespan.

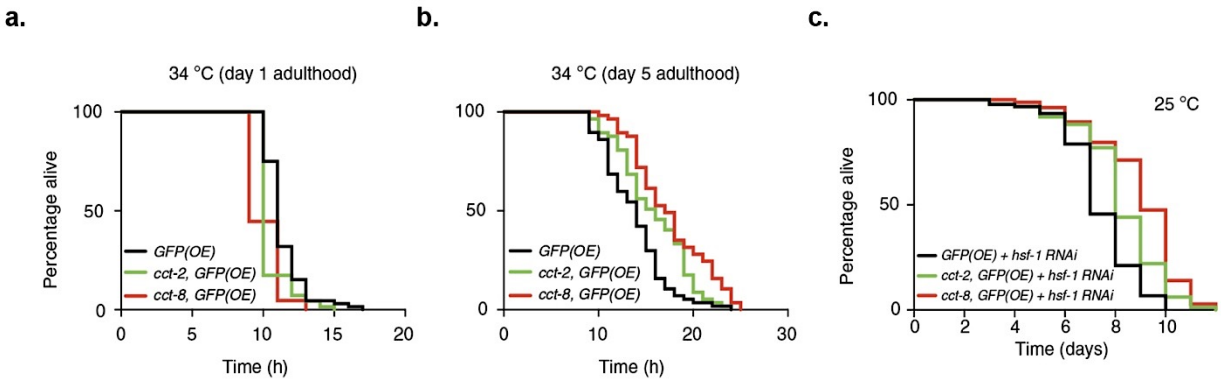

Figure 16 ***cct-8*** **determines proteotoxic stress resistance during aging. (a).** When subjected to heat stress (34°C) at day 1 of adulthood, both *cct-8(OE)* and *cct-2(OE)* worms show similar survival rates compared with the control strain (*GFP(OE)*: median= 11, n= 70/72; *cct-2, GFP(OE)*: median= 10, n= 71/72; *cct-8, GFP(OE)*: median= 9, n= 70/72). **(b)** In contrast, *cct-8(OE)* (log rank, P<0.0001) and *cct-2(OE)* (log rank, P=0.0036) worms survive longer than control strain when subjected to heat stress at day 5 of adulthood (*GFP(OE)*: median= 14, n= 57/58; *cct-2, GFP(OE)*: median= 16, n= 57/57; *cct-8, GFP(OE)*: median= 17, n= 57/57). **(c)** *hsf-1*-RNAi-treated cct(OE) worms were long-lived compared to control strain under the same treatment (log rank, P<0.0001). RNAi was initiated at day 1 of adulthood. GFP(OE) fed *hsf-1* RNAi bacteria: median= 7, n= 90/96; cct-2, GFP(OE) fed *hsf-1* RNAi bacteria: median= 8, n= 82/96; *cct-8, GFP(OE)* fed *hsf-1* RNAi bacteria: median= 9, n= 72/96.

Growing evidence suggest that the TRiC/CCT complex could be a therapeutic target in Huntington's disease (HD) (Kitamura, Kubota et al. 2006, Tam, Geller et al. 2006). For instance, simultaneous overexpression of all eight CCT subunits ameliorates polyQ-expanded HTT aggregation and neuronal death (Kitamura, Kubota et al. 2006). Intrigued by the protection that *cct-8* could confer, we tested whether increased levels of this subunit are sufficient to protect from polyQ-expanded aggregation. For this purpose, we used a *C. elegans* model that expresses polyQ peptides throughout the nervous system. In these worms, neurotoxicity correlates with increased length of the polyQ repeat and age (Brignull, Moore et al. 2006, Vilchez, Boyer et al. 2012). The neurotoxic effects can be monitored by worm motility, which is markedly reduced by the aggregation of polyQ peptides with a pathogenic threshold at a length of 35–40 glutamines (Brignull, Moore et al. 2006). We found that ectopic expression of *cct-8* reduced toxicity and improve motility of worms expressing polyQ67 either at 20°C or 25°C (Figure 17a-b). Similarly, *cct-2(OE)* also ameliorated the neurotoxic effects of polyQ67 aggregation although the impact of *cct-8* is more dramatic

(Figure 17a-b). Moreover, filter trap analysis showed that *cct-8(OE)* reduced polyQ67 aggregates without decreasing the total protein levels of polyQ67 (Figure 17c). In fact, we observed higher expression of total polyQ67 protein in *cct-8(OE)* worms probably because they are in a healthier state than controls. Taken together, these results indicate CCT8 as a novel candidate to sustain proteostasis during aging.

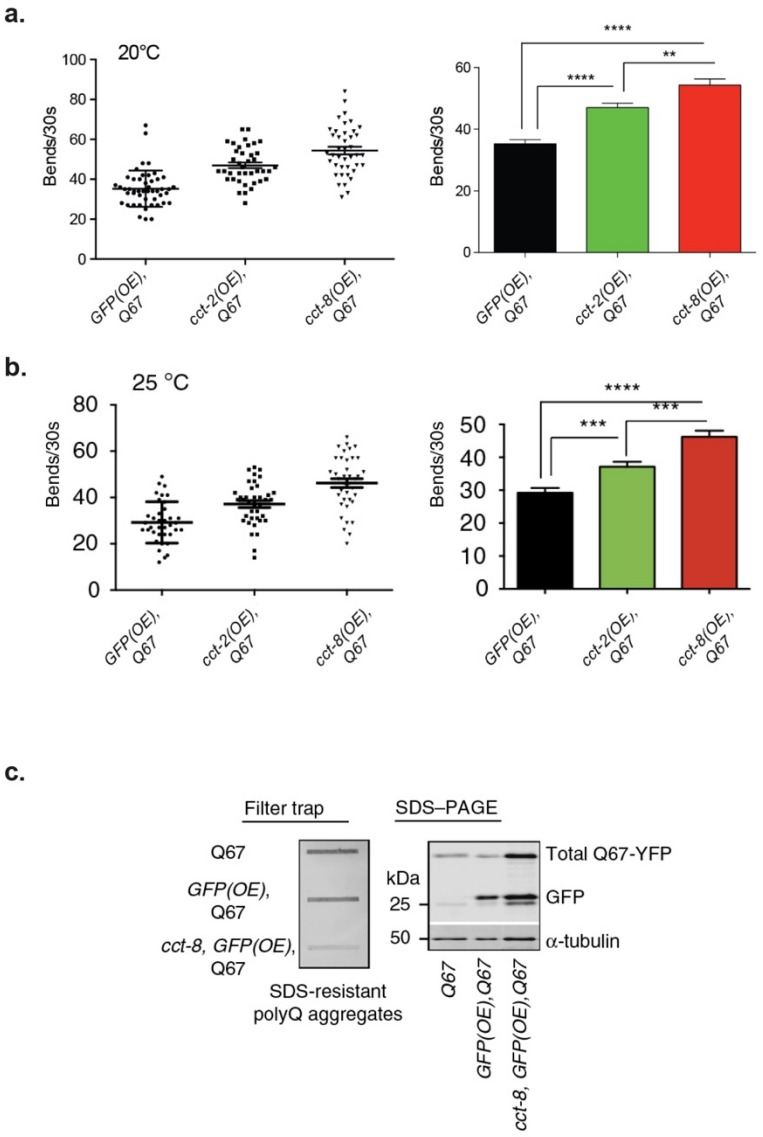

Figure 17 cct-8 ***protects from polyQ aggregation.*** **(a-b).** Ectopic expression of *cct-8* and *cct-2* improves motility in polyQ67 worms at 20 and 25°C. In the left panel, each point represents the average thrashing rate of a single 3 day-adult animal over a period of 30 s. In the right panel, bar graphs represent average ± s.e.m. of these data (*GFP(OE);Q67* (n= 37), *cct-2,GFP(OE);Q67* (n= 38), *cct-8,GFP(OE);Q67* (n= 38)). All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: ***(P<0.001), **** (P<0.0001). **(c)** Filter trap indicates that *cct-8* overexpression results in decreased polyQ aggregates (detected by anti-GFP antibody). Right panel: SDS-PAGE analysis with antibodies to GFP and α-tubulin loading control.

Longevity-promoting pathways such as reduced insulin–insulin-like growth factor (IGF) signaling (IIS) and dietary restriction (DR) delay the onset of age-related diseases associated with proteostasis dysfunction (Vilchez, Saez et al. 2014). These pathways confer increased proteome integrity and resistance to proteotoxic stress during aging (Taylor and Dillin 2011, Vilchez, Saez et al. 2014), mechanisms that contribute to lifespan extension. Thus, we hypothesized that the TRiC/CCT complex could be required for the longevity phenotype induced by IIS and DR. Notably, loss of function of TRiC/CCT complex significantly decreased longevity of long-lived IIS and DR genetic models whereas it did not affect the lifespan of wild-type worms (Figure 18a). In *C. elegans*, the aging of somatic tissues is regulated by signals from the germline (Kenyon 2010). Concomitantly, ablation of germ cells extends lifespan and induces heightened resistance to proteotoxic stress in post-mitotic tissues (Vilchez, Morantte et al. 2012, Khodakarami, Mels et al. 2015). We found that TRiC/CCT dysfunction reduces the lifespan of germline-lacking genetic worm models (Figure 18b). We have previously reported that removal of germ cells induces increase expression of key proteasome subunits in somatic tissues (Vilchez, Morantte et al. 2012). An intriguing possibility is that the germline also modulates the expression of CCT subunits in somatic tissues. Indeed, germline-lacking *C. elegans* showed up-regulation of CCT expression in somatic tissues (Figure 18d). Altogether, our data suggest that the TRiC/CCT complex could be a key determinant of longevity and proteotoxic resistance (Kitamura, Kubota et al. 2006).

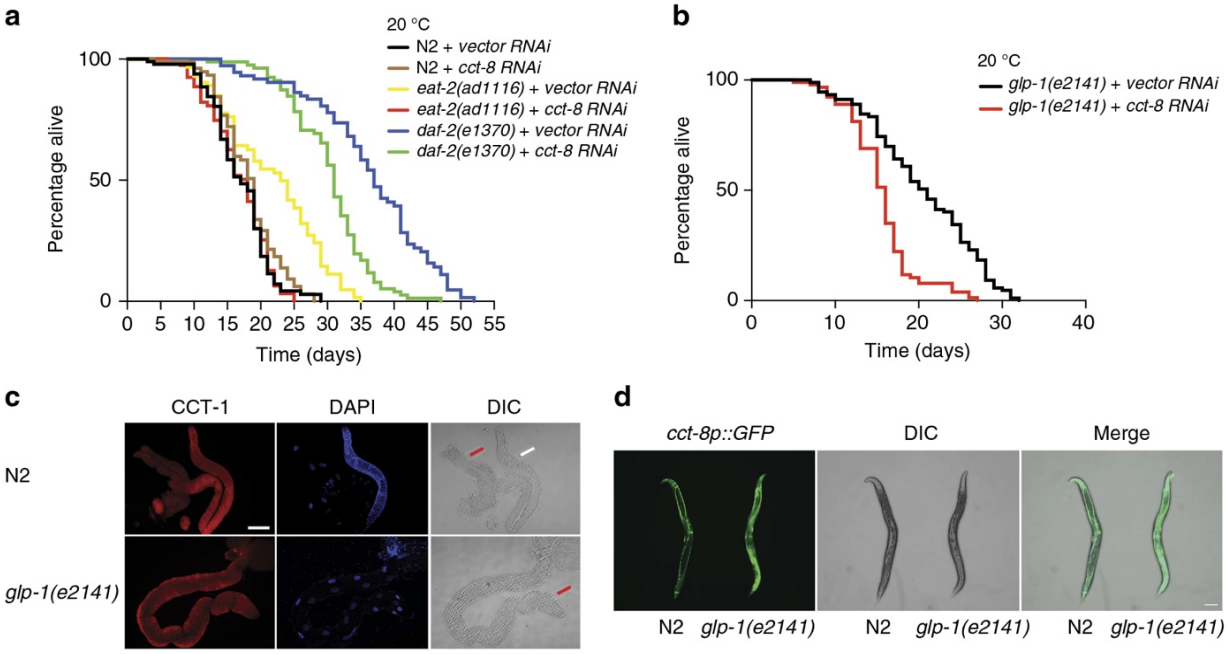

Figure 18 TRiC/CCT is required for the longevity phenotype of long-lived *C. elegans* mutants.

(a) Knockdown of *cct-8* decreases lifespan of IIS *(daf-2)* and DR *(eat-2)* long-lived mutant worms at 20°C (log rank, P<0.0001). N2 (wild-type worms) + *vector* RNAi: median= 17, n= 72/9; N2 + *cct*-8 RNAi: median= 19, n= 66/96; *eat-2(ad1116)* + *vector* RNAi: median= 23, n= 66/96; *eat-2(ad1116)* + *cct*-8 RNAi: median= 18, n= 67/96; *daf-2(e1370)* + *vector* RNAi: median= 37, n= 68/96; *daf-2(e1370)* + *cct*-8 RNAi: median= 31, n= 77/96. **(b)** Knockdown of *cct-8* decreases lifespan of germline-lacking mutant worms (log rank, Po0.0001). *glp-1*(e2141).vector RNAi: median.21, n.88/96; *glp-1*(e2141).*cct-8* RNAi: median.16, n.86/96. (**c**) Gonad and intestine immunostaining with CCT-1 antibody from wild-type and germline-lacking (*glp-1(e2141)*) worms. Long-lived germline-lacking nematodes have increased levels of CCT-1 in the intestine compared to wild-type worms. Cell nuclei were stained with DAPI. White arrow indicates gonad and red arrow indicates intestine. Scale bar represents 50 µm. **(d)** Representative images of GFP expressed under control of the *cct-8* promoter in adult wild-type and germline-lacking (*glp-1*(e2141)) worms. Scale bar represents 100 µm. In c and d the images are representative of three independent experiments. See Supplementary Data 3 for statistical analysis and replicate data of lifespan experiments.

4 Discussion

4.1 Investigating the role of PSME4 in stem cells and aging

Investigating the proteostasis of pluripotent cells in order to find regulators of stress resistance and longevity is an important and interesting field of biological research which has been emerging only in the recent years (Vilchez, Boyer et al. 2012, Vilchez, Simic et al. 2014). The knowledge gained from this line of research could not only be of crucial importance for better understanding stem cell and developmental biology, but regarding the central role of stem cells and proteostasis in the aging process (Lopez-Otin, Blasco et al. 2013) could pave the way towards development of novel therapies with wide application against age-associated diseases. In this work, we concentrated on the UPS system and the chaperome network, two of the main proteostasis components in hESCs.

The ubiquitin proteasome system is the major proteolytic system in eukaryotic cells (Meiners, Heyken et al. 2003). Previous findings showed that hESCs exhibit enhanced assembly of 26S/30S active proteasomes induced by increased levels of the 19S subunit PSMD11/RPN-6 (Vilchez, Boyer et al. 2012, Vilchez, Morantte et al. 2012). In addition to regulation by 19S, the core particle can be activated by other regulatory particles such as PSME4 (also known as the proteasome activator 200, PA200). PSME4 is a monomeric protein of 250 kDa and in contrast to the 19S regulatory particle; PSME4 can activate the core proteasome without having ATPase or ubiquitin binding properties. PSME4 can form hybrid complexes in which this protein binds to one end of the 20S proteasome and the 19S to the opposite end. PSME4 has been suggested to be essential for spermatogenesis and DNA repair, but the precise biological functions remain controversial and poorly understood (Savulescu and Glickman 2011). Therefore, understanding its impact on hESCs function not only has implications in hESC function but also in defining the cellular role of this protein.

We discovered that expression of PSME4 is remarkably elevated in hESCs compared to differentiated cells of ectodermal or mesodermal lineages. On the other hand, It is interesting that the expression of PSME4 is dramatically increased upon endodermal differentiation, which might hint towards special function of this protein in the endoderm lineage. Moreover, regulation of PSME4 expression is reprogrammable (Figure 1a-e).

However, the stem cells were not affected by downregulation of PSME4 (Figure 2). This was demonstrated by the intact expression of major pluripotency markers such as *OCT4*, *NANOG* and *SOX2*, and absence of any difference in expression of difference lineage markers such as *AFP* and *MSX* in PSME4 KD hESCs. There are some significant differences in expression of a subset of markers, but for all of these markers, this difference could only be observed in one of the KD lines. Based on the observation that the PSME4 knockdown is quite efficient in both cell lines infected with either shRNA (Figure 1 e-f), and the capability of downregulated PSME4 cells to differentiate into endodermal or neural lineages (Figure 2b-c) this could be interpreted as off target side effects of the lentiviral transduction than a biologically relevant consequence of lesser PSME4 levels. The reasons behind the lack of any obvious consequence upon PSME4 downregulation could be diverse. It is known that cells possess complex adaptive mechanisms to compensate for interference in the proteasome (Meiners, Heyken et al. 2003). To prevent this, we also used transient lentiviral infections, and checked for different marker expression and differentiations, but could not detect any changes (data not shown). It is therefore plausible that other genes can compensate for lack of PSME4 under normal conditions. PSME4 is mainly localized in the nucleus and has been suggested to have a role in DNA repair (Ustrell, Hoffman et al. 2002). Stem cells have been shown to have enhanced DNA damage repair mechanisms such as error free homologous recombination (HR) and non-homologous end joining (NHEJ) (Durante 2011). This leads to lesser accumulation of spontaneous mutations compared to somatic cells, and the capability to maintain self-renewal and genomic stability (Maynard, Swistowska et al. 2008, Frosina 2010). However, previous studies suggest that murine and human neural stem cell precursors demonstrate higher sensitivity to ionizing radiation (Nowak, Etienne et al. 2006, Panagiotakos, Alshamy et al. 2007). Interestingly, PSME4 has been shown to enhance the post-glutamyl activity of the proteasome and regulate cellular resistance to IR (Blickwedehl, Agarwal et al. 2008) (Blickwedehl, Olejniczak et al. 2012). It could be therefore hypothesized that higher levels of PSME4 in hESCs provides them with more protection against IR induced DNA damage. Taken the impact of PSME4 in response to DNA damage (Ustrell, Hoffman et al. 2002), further studies are required to shed light on lineage specific role of this protein in DNA damage repair in stem cells. Moreover, it could be interesting to challenge the hESCs with different stress conditions and check if their function is affected. It is also interesting to knockdown/overexpress PSME4 in cells derived from patients suffering from protein misfolding diseases and to investigate the effects, as it has been suggested that PSME4 is mainly involved in degradation of peptides and unstructured proteins (Tar, Dange et al. 2014).

Taken together, from the experiments shown here it could be concluded that PSME4 is not vital for hESCs under normal conditions and might only be responsible for specific functions in particular lineages and/or under specific conditions.

Investigating the biology of PSME4 in *C. elegans* faces an extra hurdle: the gene has two orthologues in worms. In this work, we mainly concentrated on *C14C10.5*, which exhibits more similarity to human PSME4 in the amino acid sequence (Figure 3a). Maintaining a balanced proteostasis is crucial for healthy ageing and longevity. We therefore asked whether manipulation of *psme4* could lead to accumulation of protein damage and affect lifespan. By using RNAi and creating extrachromosomal arrays we studied the impact of psme4 downregulation or overexpression on longevity and stress resistance in *C. elegans*. We were not able to detect any significant changes in the lifespan of worms, neither under normal growth conditions (20°C) or mild proteotoxic stress (25°C) (Figure 3b-f).

Lack of any spectacular phenotypes in hESCs and *C.elegans* lifespan under normal conditions motivated us to study the impact of psme4 for stress resistance. Interestingly, we observed that the worms downregulated for psme4 live longer than control worms. Reversely, overexpression of psme4 lead to a minor, but significant, decrease in heat stress resistance of worms (Figure 4). This observation could be because psme4 does not play a major role in the clearance of heat-damaged proteins, and its presence in the assembled proteasomes shifts the balance towards proteasome species, which are inactive against heat stress, thus reducing the overall survival of the organism. We went further to ask whether psme4 might be important for resistance against oxidative stress. Whereas downregulation of psme4 did not affect the survival of worms, we observed a small yet significant increase in the survival of one of the overexpression lines. The difference between the two lines can be explained by the fact that these lines are not integrated, and overexpression levels might vary to some extent among different lines. Taken the dramatic changes in protein oxidation levels during differentiation, and the role of 20S proteasome in clearance of this damaged proteins (Hernebring, Brolén et al. 2006), we asked whether psme4 could play a role in clearance of oxidized proteins in this conditions. As we observed a major increase in PSME4 expression in early endoderm progenitors, we performed OxyBlot analysis both in hECSs and endoderm progenitors (Figure 4e). Although we once observed an increase in the levels of oxidized proteins in endoderm

cells downregulated for PSME4 (Data not shown) this effect was not observed in any of the following experimental replicates, and thus could not be confirmed.

Interestingly, a recent study hints towards a role for Psme4 in antioxidant response (Huang, Haratake et al. 2016). In this study, it is suggested that deletion of Psme4, together with another proteasome activator PA28γ (or PSME3, which also shows elevated expression in hESCs compared to differentiated counterparts) leads to complete male infertility in mice. These double knockout (dKO) sperms showed compromised functionality and motility, as well as reduced proteasome activity and accumulation of oxidative damage (Huang, Wang et al. 2014). This confirms our hypothesis that lack of PSME4 alone could be somehow compensated, by other proteostasis mechanisms in the cells, and the cells could cope with its absence, unless confronted with specific stress, or additional burden of missing an extra proteasome subunit.

4.2 Investigating the role of TRiC/CCT in stem cells and aging

Despite the recent studies, which link the UPS in hESCs and organismal aging, a comprehensive study of the role of molecular chaperones in stem cell biology has yet been missing. By using quantitative proteomics and RNA sequencing, we compared the expression levels of components of the chaperome network in hESCs to differentiated cells. At the protein level, for instance, we found that 36 out of 122 identified chaperome components decrease during differentiation into NPCs. In contrast, 27 chaperome components were increased during neural differentiation. Taken the important role of TRiC/CCT complex in cellular ptoteostasis, we concentrated in studying the role of this huge molecular machine in stem cell biology. Our findings establish enhanced assembly of the TRiC/CCT complex as an intrinsic characteristic of pluripotent stem cells. This increased assembly correlates with an up-regulation of several CCT subunits. Notably, hESCs are able to maintain high expression of CCT subunits during unlimited proliferation. However, hESCs lose their high levels of CCT subunits upon differentiation and this decline is already significant in multipotent cells (*e.g.*, NPCs) before terminal differentiation (Figure 5). Therefore, enhanced TRiC/CCT assembly could be linked to the immortal and pluripotent characteristics of hESCs/iPSCs. Indeed, we found that slight dysfunctions of the TRiC/CCT complex induce a decrease in pluripotency markers and, concomitantly, an increase in differentiation markers. Defining the

mechanisms by which increased TRiC/CCT regulates hESC identity could open a new door to a better understanding of pluripotency and differentiation. Although the exact mechanisms are still unknown, the TRiC/CCT complex could impinge upon hESC function in several (and non-exclusive) manners. TRiC/CCT assists the folding of a significant percentage of nascent proteins such as actin and tubulin (Gao, Thomas et al. 1992, Yam, Xia et al. 2008). Thus, one possibility is that enhanced TRiC/CCT assembly is required for the proper folding of specific regulatory or structural proteins involved either in maintenance of hESC identity or generation of healthy differentiated cells. Interestingly, a study performed in calreticulin$^{-/-}$ mouse ESCs showed a downregulation in the expression of *Cct2*, *Cct3* and *Cct7* (Faustino, Chiriac et al. 2010) in these cells. Calreticulin, a chaperone that binds to misfolded proteins preventing their export from the endoplasmic reticulum, is essential for cardiac development in mice (Mesaeli, Nakamura et al. 1999) and required for proper myofibril formation during cardiomyocyte differentiation of mouse ESCs *in vitro* (Li, Puceat et al. 2002). Since the TRiC/CCT complex regulates folding and actin dynamics, the downregulation of Cct subunits in calreticulin$^{-/-}$ mouse ESCs could forecast the myofibrillar disarray observed in their cardiomyocytes counterparts (Faustino, Chiriac et al. 2010). As TRiC/CCT also regulates the aggregation of misfolded and damaged proteins, increased TRiC/CCT levels could be linked to the biological purpose of pluripotent stem cells and their intrinsic properties such as high global protein synthesis, proliferation rates and immortality.

hESCs exhibit a distinct cell cycle signature and high proliferation rates has been shown to be essential for maintenance of hESC identity as well as reprogramming efficiency (Ruiz, Panopoulos et al. 2011). CCT is responsible for folding of many cell cycle components, such as Plk1, whose activity is required for the mammalian cells to enter mitosis (Liu, Lin et al. 2005). Thus CCT could also be an essential factor in regulating cell cycle and proliferation rate of hESCs. Increased levels of translation is another characteristic of ESCs in comparison to differentiated cells (You, Park et al. 2015). CCT is also directly involved in regulating the translation rate, at least by regulating the folding of three subunits of the translation initiation factor eIF3 (Roobol, Roobol et al. 2014). Telomere length is another key factor, which is essential for maintaining the proliferative potential and replicative immortality of stem cells (Allen and Baird 2009). ESCs have increased telomerase activity, and this higher activity is not only required for stem cell self-renewal and extended lifespan, but also enhances stress resistance by influencing stress response genes (Armstrong, Saretzki et al. 2005). CCT has recently be discovered to be a critical regulator of telomerase trafficking

by folding the telomerase cofactor TCAB1 (Freund, Zhong et al. 2014). Lack of CCT was shown to cause mislocalization of telomerase, and failure of telomere elongation due to lack of TCAB1 (Freund, Zhong et al. 2014). Together, it could be concluded that various aspects of stem cell biology are directly regulated by CCT, and lack of CCT activity could have more detrimental consequences for stem cells compared to differentiated ones.

Given the ability of hESCs to replicate indefinitely and generate every other cell type in the body, the TRiC/CCT complex could be a key quality control mechanism to maintain a global intact proteome for either self-renewal or the generation of progenitor cells. In this context, the accumulation of damaged and misfolded proteins caused by proteostasis defects may affect hESCs function and immortality. With the asymmetric divisions invoked by these cells, the passage of damaged proteins to progenitor cells could also compromise development and organismal aging. In support of this hypothesis, we found that the TRiC/CCT complex determines the ability of iPSCs to maintain proteostasis of mutant huntingtin.

Another important question is how hESCs achieve their high TRiC/CCT levels. Interestingly, specific subunits such as CCT4 are relatively more abundant compared to CCT1 in hESCs whereas this proportion is reversed in NPCs (Figure 7). Therefore, these subunits could become limiting assembly factors during differentiation. Despite decreasing during neural differentiation, other CCT subunits (*i.e.*, CCT8 and CCT2) were more abundant relatively to the rest of subunits in both hESCs and NPCs suggesting that they could be activators of TRiC/CCT assembly rather than limiting factors. Indeed, increasing the expression of CCT8, the most abundant subunit, triggers TRiC/CCT assembly regardless the levels of other subunits such as CCT1 in both mammalian cells and *C. elegans*. Further studies will be required to understand how CCT8 promotes TRiC/CCT assembly. A fascinating hypothesis is that CCT8 could act as a scaffold protein that triggers the interaction between the different subunits.

Our findings in human pluripotent stem cells led us to identify CCT8 as a powerful candidate to sustain proteostasis during organismal aging. The somatic induction of TRiC/CCT assembly via CCT8 overexpression extends lifespan and protects from proteotoxic stress in *C. elegans*. Notably, somatic CCT8 overexpression can partially protect this nematode from knockdown of *hsf-1*, a transcription factor that coordinates cellular protein-

misfolding responses. The precise mechanism by which the TRiC/CCT complex ameliorates the detrimental effects triggered by loss HSF1 is not yet understood. In response to proteotoxic stress, HSF1 binds heat shock elements (HSE) in the promoters of target genes and triggers their expression (Akerfelt, Morimoto et al. 2010). In mammalian cells, all the CCT subunits contain HSE and are transcriptionally activated by HSF1 (Kubota, Matsumoto et al. 1999). Thus, ectopic expression of CCT8 could ameliorate the effects induced by a decrease of CCT subunits upon HSF1 knockdown. In addition, a direct regulatory interaction between TRiC/CCT activity and induction of proteotoxic-stress response by HSF1 has been recently reported (Neef, Turski et al. 2010, Neef, Jaeger et al. 2014). HSF1A, a chemical activator of HSF1, binds to the TRiC/CCT complex and inhibits its folding activity (Neef, Turski et al. 2010, Neef, Jaeger et al. 2014). Both the inactivation of TRiC/CCT complex by HSF1A or its depletion by loss of CCT subunits induce HSF1 activity (Neef, Jaeger et al. 2014). Since TRiC/CCT chaperonin interacts with HSF1(Neef, Jaeger et al. 2014), TRiC/CCT could have a direct repressor role in regulating HSF1 (Neef, Jaeger et al. 2014). However, a decrease in TRiC/CCT activity mediated by either HSF1A or knockdown of CCT subunits can also lead to the accumulation of misfolded proteins that trigger the HSF1-induced proteotoxic response (Neef, Jaeger et al. 2014). In support of this hypothesis, our results suggest that increased TRiC/CCT assembly induced by CCT8 reduces the accumulation of misfolded proteins and, therefore, ameliorates the deleterious impact of reduced HSF1-mediated signaling.

During organismal aging, loss of proteostasis in somatic tissues could contribute to the late onset of age-related diseases such as HD (Lopez-Otin, Blasco et al. 2013, Brehme, Voisine et al. 2014). Hence, the decrease in the levels of several CCT subunits during human brain aging (Brehme, Voisine et al. 2014) may induce TRiC/CCT dysfunction, proteotoxic stress and neurodegeneration. In support of this hypothesis, knockdown of CCT subunits hastens aggregation of mutant HTT and worsens HD-related changes in HD models (Nollen, Garcia et al. 2004, Kitamura, Kubota et al. 2006, Tam, Geller et al. 2006, Brehme, Voisine et al. 2014). Previous studies have shown that simultaneous overexpression of all eight CCT subunits ameliorates polyQ-expanded HTT aggregation and neuronal death (Kitamura, Kubota et al. 2006). Furthermore, overexpression of CCT1 remodels the morphology of HTT aggregates and reduces neurotoxicity (Tam, Geller et al. 2006). Although CCT1 overexpression does not increase TRiC/CCT assembly, this subunit modulates the interaction between HTT and TRiC/CCT complex (Tam, Geller et al. 2006). Moreover, a recent study

showed that both CCT3 and the apical domain of CCT1 are able to enhance the transport of brain-derived neurotrophic factor (BDNF) and lysosomes in axons and rescue striatal atrophy in HD models by reducing the mutant HTT (Zhao, Chen et al. 2016). Since CCT8 is sufficient to increase TRiC/CCT assembly, this subunit could be a candidate to correct deficiencies in age-related diseases associated with proteostasis dysfunction. Indeed, we found that overexpression of CCT8 can protect from the accumulation of polyQ-expanded proteins. Recently, a link between CCT8 and mutant HTT expression was observed in an integrated genomics and proteomics study of knock-in HD mouse models expressing the human *HTT* exon1 carrying different CAG lengths (polyQ20, Q80, Q92, Q111, Q140 or Q175) (Langfelder, Cantle et al. 2016). Notably, highly expanded-polyQ *HTT* exon1 (Q175) induces a significant increase of CCT8 at both transcript and protein levels in the striatum of young mice (6-months) whereas other subunits were not altered (Langfelder, Cantle et al. 2016). In contrast, CCT8 induction was not observed in knock-in mice expressing mutant HTT with lower than 175 polyQ repeats. These findings suggest that up-regulation of CCT8 could be a compensatory mechanism to protect from polyQ aggregation.

While expression of CCT subunits decrease during both differentiation and biological aging, our results indicate that mimicking proteostasis of hESCs by modifying either the chaperome or proteasome network (Vilchez, Boyer et al. 2012) delays the aging of somatic cells and extends organismal lifespan. Given the link between sustained proteostasis in somatic cells and healthy aging, our findings raise an intriguing question: why the levels of CCT8 decrease during differentiation if this subunit could have beneficial effects on organismal longevity and proteotoxic stress resistance? In this regard, it is important not to diminish potential detrimental effects of mimicking proteostasis of ESCs in somatic tissues. For instance, cancer cells and ESC not only share their immortality features but also increased proteostasis nodes such as proteasome activity and specific chaperones (Whitesell and Lindquist 2005, Saez and Vilchez 2014, Vilchez, Simic et al. 2014). Proteasome and chaperone levels in cancer cells are consistent with the special requirements of these cells, such as elimination of aberrant proteins. Interestingly, the expression of CCT8 is increased in gliomas and hepatocellular carcinoma whereas its knockdown induces a decrease in the proliferation and invasion capacity of these cells (Huang, Wang et al. 2014, Qiu, He et al. 2015). Similarly, other CCT subunits have been linked to cancer including colorectal carcinoma and breast cancer (Boudiaf-Benmammar, Cresteil et al. 2013). Whereas proteasome inhibitors and interventions of the chaperone network have been suggested as potential strategies for anticancer therapy

(Whitesell and Lindquist 2005, Rappa, Farina et al. 2012), an abnormal activation of these mechanisms in somatic dividing cells could have the opposite effect inducing their abnormal proliferation. However, although the TRiC/CCT complex is important for the proliferation of cancer cells, this chaperonin is also required for the proper folding of p53 and, therefore, promotes tumor suppressor responses (Trinidad, Muller et al. 2013). Thus, the TRiC/CCT complex could be an important factor to avoid misfolding of tumor suppressors and the increase incidence of cancer during the aging process. Besides its link with cancer, other factors could explain the decline of CCT subunits during differentiation. In support of the disposable soma theory of aging (Kirkwood 1977), a fascinating hypothesis is that downregulation of the TRiC/CCT complex during differentiation is part of an organismal genetic program that ensures a healthy progeny whereas somatic tissues undergo a progressive demise in their homeostasis and function. Due to the limitation of nutrients in nature, organisms divide the available metabolic resources between reproduction and maintenance of the non-reproductive soma. Evolutionary pressure has been theorized to force a re-allocation of the resources to prevent or eliminate damage to the germline and progeny, while little resources are placed on the maintenance of somatic cells (Kirkwood 1977). In support of this hypothesis, signals from the germline modulate the aging of somatic tissues and removal of the germline extends lifespan (Khodakarami, Mels et al. 2015). Interestingly, our results indicate that germline-lacking worms exhibit increased levels of TRiC/CCT in post-mitotic cells.

Conclusion

Collectively, we underscore the importance of defining proteostasis of hESCs to uncover novel mechanisms of organismal healthspan extension. Besides CCT subunits, other chaperome components also demonstrate differential expression during differentiation and, therefore, could be interesting targets to be studied in the context of aging. In addition, it will be fascinating to explore whether and how other proteostasis components such as autophagy, HSR, UPR^{ER} and UPR^{mt} are also divergent in hESCs, whether they impact stem cell pluripotency and self-renewal and if mimicking these mechanisms in somatic cells, tissues or whole organisms can extend organismal healthspan or ameliorate disease related pathologies.

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie- abgesehen von unten angegebenen Teilpublikationen- noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

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Amirabbas Khodakarami

Geburtsdatum und -ort. 21.09.1988- Hamedan, Iran
Anschrift: Höninger Weg 206, 50969 Cologne, Germany
Tel.: +491776432139 **E-mail:** khodakaa@uni-koeln.de

Hochschulbildung

Sep 2013-	**PhD in Genetik** CECAD Research Institute, Cologne, Germany Thesis: Impact of the chaperone network on stem cells and aging Supervisor: Dr. David Vilchez
Sep 2011-Sep 2013	**MSc. Biological Sciences (Genetik)** University of Cologne, Germany Thesis: The role of CLUH in regulating mitochondrial distribution Supervisor: Prof. Dr. Elena Rugarli
Sep 2006-Sep 2010	**BSc. Biologie** Shahid Beheshti University, Tehran, Iran Thesis: Bioremediation of oil sludge contaminated soil Supervisor: Dr. Dariush Minai-Tehrani

Publikationen

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