UNIVERSIDADE DE SÃO PAULO

Instituto de Ciências Biomédicas

CAMILA FELIX DE LIMA FERNANDES

Estudo do papel da STIP1 e potenciais fatores associados à rede de proteostase na regulação da pluripotência

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CAMILA FELIX DE LIMA FERNANDES

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Orientadora: Marilene Hohmuth Lopes

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CAMILA FELIX DE LIMA FERNANDES

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Certificamos que o Protocolo CEP-ICB nº **945/2018** referente ao projeto intitulado: "***Estudo do perfil da expressão gênica de células-tronco embrionárias murinas haploinsuficientes para STI1 na manutenção do estado pluripotente***" sob a responsabilidade de ***Camila Felix de Lima Fernandes*** e orientação do(a) Prof.(a) Dr.(a) ***Marilene Hohmuth Lopes,*** do Departamento de Biologia Celular e do Desenvolvimento, foi analisado pela **CEUA** - Comissão de Ética no Uso de Animais e pelo **CEPSH** – Comitê de Ética em Pesquisa com Seres Humanos, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da Lei nº 11.794, de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº 466/2012.

São Paulo, 21 de março de 2018.

Profa. Dra. **Luciane Valéria Sita**
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Profa. Dra. **Camila Squarzoni Dale**
Coordenadora CEPSH ICB/USP

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If you wish to make an apple pie from scratch,
you must first invent the universe

— Carl Sagan

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Thesis organization

This Thesis was written in English (except for the sections Acknowledgements and "Resumo") and organized into four chapters. The chapters are preceded by a **General Introduction** that presents the theoretical basis that led to the formulation of this research proposal, described in the **Aims** section. The chapters are organized into sub-sections of the *Rationale* of that specific topic, depicting the literature background for that investigation, the *Materials and Methods* of the following experiments, *Results and Discussion*, *Conclusions* and *References*. Specifically, the four chapters explore different aspects of this research: **Chapter I** corresponds to a published literature review that addresses the function of chaperone proteins and other mechanisms of protein homeostasis in the control of pluripotency; **Chapter II** corresponds to an original manuscript submitted for publication, which brings together data showing the importance of the stress-inducible protein 1 in the pluripotent phenotype of mouse embryonic stem cells; In **Chapter III** we explore the spectrum of pluripotent states under the perspective of the possible contributions of the stress-inducible protein 1 to the manifestations of these phenotypes; and lastly, **Chapter IV** explores the extent of evolutive maintenance of the observations from mouse pluripotent cells in human pluripotent cells. We conclude this Thesis with the **Conclusions** and **Perspectives**, and the **References** for the General Introduction. In the **Annexes**, the reader can find the list of publications originating from the research carried out during this doctorate.

List of abbreviations

17-AAG: 17-allylamino-17-dimethoxy-geldanamycin

AKT1: RAC-alpha serine/Threonine-protein Kinase

ANOVA: Analysis of variance

ASF1 (A/B): Anti-Silencing Function 1 Histone Chaperone (A/B)

ATCC: American Type Culture Collection

ATRX: ATRX Chromatin Remodeler

BAC: Bacterial artificial chromosome

BAG3: BAG family molecular chaperone regulator 3

BMP4: Bone morphogenetic protein 4

c-MYC: MYC Proto-Oncogene/BHLH Transcription Factor

C-terminal: Carboxy-terminal domain

CAF-1 (A/B): Chromatin assembly factor 1 (subunit A/B)

CBX4: Chromobox 4

CBX6: Chromobox 6

CC3: Cleaved caspase-3

CDK1/CDC2: Cyclin Dependent Kinase 1/ Cell Division Cycle 2

cDNA: Complementary DNA

CDS: Coding Sequence

CER1: Cerberus 1/DAN Family BMP Antagonist

CMA: Chaperone-mediated autophagy

COP9: Constitutive photomorphogenesis 9 signalosome

COPS2: COP9 Signalosome Subunit 2

CTEm: Células-tronco embrionárias murinas

CTPs: Células-tronco pluripotentes

CTSA: Cathepsin A/Lysosomal protective protein

DAX1/ NR0B1: Nuclear receptor subfamily 0 group B member 1/ DSS-AHC Critical Region On The X Chromosome Protein 1

DAXX: Death Domain Associated Protein

DEGs: Differentially expressed genes

DEPs: Differentially expressed proteins

DNAJA2: DnaJ homolog subfamily A member 2

DNAJB6: DnaJ homolog subfamily B member 6

DNAJC3: DnaJ homolog subfamily C member 3

DP1: Dipeptide domain 1

DP2: Dipeptide domain 2

DUBs: Deubiquitinases

E: Embryonic day

EIF4E: Eukaryotic translation initiation factor 4E

EIF4G: Eukaryotic translation initiation factor 4G

EIF6: Eukaryotic translation initiation factor 6

EOMES: Eomesodermin homolog

ERK: Extracellular signal-regulated kinase

ERK1/MAPK3: Extracellular Signal-Related Kinase 1/Mitogen-activated protein kinase 3

ESCs: Embryonic stem cells

ESSRB: Estrogen Related Receptor Beta/Steroid hormone receptor ERR2

ETV5: ETS translocation variant 5

FACT: Facilitates Chromatin Transcription

FBXO15: F-box only protein 15

FBXW7: F-box/WD repeat-containing protein 7

FBXW8: F-box/WD repeat-containing protein 8

FGF2: Fibroblast growth factor 2

FGF5: Fibroblast growth factor 5

FOXA2: Hepatocyte nuclear factor 3-beta

FOXC1: Forkhead box protein C1

FOXF1: Forkhead box protein F1

FOXO4: Forkhead box protein O4

GATA4: GATA Binding Protein 4

GATA6: GATA Binding Protein 6

GBX2: Homeobox protein GBX-2

GMEM: Glasgow's Minimum Essential Medium

GO-BP: Gene Ontology - Biological processes

GO-CC: Gene Ontology - Cellular components

GO-MF: Gene Ontology - Molecular functions

GSK3: Glycogen synthase kinase 3

HDAC: Histone deacetylase

HECT: Homologous to the E6-AP Carboxyl Terminus

HEK293T: Human embryonic kidney 293 cells, SV40 T-antigen

hESCs: Human Embryonic Stem Cell

HIF1α: Hypoxia Inducible Factor 1 Alpha

hiPSCs: Human Induced Pluripotent Stem Cells

HIRA: Histone Cell Cycle Regulator

HMGA2: High Mobility Group At-Hook 2

HNF4A: Hepatocyte Nuclear Factor 4 Alpha

HOP: Heat Shock Organizing Protein

HSE: Heat Shock Element

HSF1: Heat Shock Transcription Factor 1

HSP10: 10kDa Heat Shock Protein

HSP100: 100kDa Heat Shock Protein/Clp Subfamily

HSP110: 110kDa Heat Shock Protein

HSP22/HSPB8: Heat Shock 22kda Protein 8/Heat Shock Protein Family B (Small) Member 8

HSP25/HSP27/HSPB1: Heat Shock 27kDa Protein 1/Heat shock protein family B (Small) Member 1

HSP32/HMOX1: Heat Shock Protein, 32kDa /Heme Oxygenase (Decycling) 1

HSP40: 40kDa Heat Shock Protein/Dnaj-Like Proteins

HSP47/SERPINH1: 47kda Heat Shock Protein /Serpin Family H Member 1

HSP60/HSPD1: 60kDa Heat Shock Protein/Heat Shock Protein Family D (HSP60) Member 1

HSP70: 70kDa Heat Shock Protein

HSP70A9: 70kda Heat Shock Protein A9 Variation

HSP90: 90kDa Heat Shock Protein

HSP90B1: Heat Shock Protein 90 Beta Family Member 1

HSPA1A: Heat Shock 70kDa Protein 1A

HSPA1B: Heat Shock 70kDa Protein 1B

HSPA4: Heat shock Protein Family A (HSP70) Member 4

HSPA5: Heat Shock Protein Family A (HSP70) Member 5

HSPA8: Heat Shock Protein Family A (HSP70) Member 8

HSPA9A: Heat Shock Protein Family A (HSP70) Member 9A

HSPB7: Heat Shock Protein Family B (Small) Member 7

HSP90AB1/HSPCB: Heat Shock Protein 90 Alpha Family Class B Member 1

HSPs: Heat Shock Proteins

ICM: Inner Cell Mass

IDHs: Isocitrate Dehydrogenase Genes

IL6: Interleukin-6

iPSCs: Induced Pluripotent Stem Cells

hiPSCs: Human Induced pluripotent Stem Cells

JAKs: Janus kinases

KD: Knockdown

KI67/MKI67: Marker of Proliferation Ki-67/Antigen Identified by Monoclonal Antibody Ki-67

KLF2: KLF Transcription Factor 2

KLF4: KLF Transcription Factor 4

KLF5: KLF Transcription Factor 5

KO: Knockout

L1TD1: LINE1 Type Transposase Domain Containing 1

LEFTY1: Left-right Determination Factor 1

LEFTY2: Left-right Determination Factor 2

LIF: Leukaemia Inhibitory Factor

LIFR: Leukaemia Inhibitory Factor Receptor

MAPK: Mitogen Activated Kinase-Like Protein

MEFs: Mouse Embryonic Fibroblasts

MEK: Mitogen-activated Protein Kinase

mESCs: Mouse Embryonic Stem Cells

MG132: Carbobenzoxy-l-leucyl-l-leucyl-l-leucinal/Proteasome inhibitor

N-terminal: Amino-terminal Domain

NANOG: Homeobox Transcription Factor Nanog

NEBs: Neurogenic EBs

NLS: Nuclear localization signal

NPM2: Nucleoplasmin-2

NPM3: Nucleoplasmin-3

NRF2/NFE2L2: Nuclear Factor, Erythroid 2 Like 2/NFE2 Like BZIP Transcription Factor 2

NT: Non-target

OE: Overexpression

OIP5/MS18B: Opa Interacting Protein 5/Protein Mis18-Beta

ORA: Overrepresentation Analysis

OTX2: Homeobox protein OTX2

p21/CDKN1A: P21Cip1/Waf1/Cyclin Dependent Kinase Inhibitor 1A

p23: Co-chaperone Protein p23-1

p53/TP53: Cellular tumour antigen p53

PAX6: Paired Box Protein Pax-6

PBS: Phosphate Buffered Saline

PCA: Principal Component Analysis

PDMCs: Placenta-Derived Multipotent Cells

PECAM1: Platelet Endothelial Cell Adhesion Molecule

pH2AX/γH2AX: Phosphorylated Variant Histone H2A Family Member X

PHB: Prohibitin

PIAS1: Protein Inhibitor of Activated STAT 1

PN: Proteostasis Network

POU3F1/OCT6: POU Class 3 Homeobox 1/Octamer-Binding Transcription Factor 6

POU5F1/OCT4: POU Class 5 Homeobox 1/Octamer-Binding Protein 4

PRC1: Protein Regulator of Cytokinesis 1

PRC2: Protein Regulator of Cytokinesis 2

PRDM14: PR Domain Zinc Finger Protein 14

PrP^{C}: Cellular Prion Protein

PSCs: Pluripotent Stem Cells

PSMD11: Proteasome 26S Subunit, Non-ATPase 11

PSMD14: Proteasome 26S Subunit, Non-ATPase 14

PSME3: Proteasome Activator Subunit 3

RB1: Retinoblastoma-associated Protein, Transcriptional Corepressor 1

RBPJ: Recombination Signal Binding Protein for Immunoglobulin Kappa J Region

rESCs: Rabbit Embryonic Stem Cells

REX1/ZPF42: REX1 Transcription Factor/Zinc Finger Protein 42 Homolog

RNA-seq: RNA Sequencing

RONIN/THAP11: THAP domain-containing protein 11

RT-qPCR: Real-time quantitative PCR

RUNX1: Runt-related transcription factor 1

SALL2: Spalt Like Transcription Factor 2

SALL4: Spalt Like Transcription Factor 4

scRNA-seq: Single-cell RNA Sequencing

SET: SET Nuclear Proto-Oncogene

SET7/SETD7: SET Domain Containing 7, Histone Lysine Methyltransferase

SHH: Sonic Hedgehog Signaling Molecule

shRNA: Short-hairpin RNA

sHSPs: Small Heat Shock Proteins

SMAD1: SMAD Family Member 1

SMC: Smooth Muscle Cells

SOX17: SRY-Box Transcription Factor 17

SOX2: SRY-Box Transcription Factor 2

SOX3: SRY-Box Transcription Factor 3

SPT16: Suppressor of Ty 16 Homolog

SPT6: SPT6 Homolog, Histone Chaperone and Transcription Elongation Factor

SSEA1: Stage-Specific Embryonic Antigen 1

SSEA3: Stage-Specific Embryonic Antigen 3

SSEA4: Stage-Specific Embryonic Antigen 4

SSRP1: Structure Specific Recognition Protein 1, FACT complex subunit

STAT3: Signal Transducer and Activator of Transcription 3

Stella/DPPA3: Developmental Pluripotency-associated Protein 3

STIP1: Stress-inducible Protein 1

TBB2A: Tubulin Beta-2A Chain

TBX3: T-Box Transcription Factor 3

TFCP2L1: Transcription Factor CP2 Like 1

TFs: Transcription Factors

TGF-β: Transforming Growth Factor Beta

TH2A: Proallergic type 2 helper T Cells

TPR: Tetratricopeptide domains

TPR1: Tetratricopeptide domain 1

TPR2A: Tetratricopeptide domain 2A

TPR2B: Tetratricopeptide domain 2B

TRIM8: Tripartite Motif Containing 8

TUBB2A: Tubulin beta-2A chain

UBR5: Ubiquitin Protein Ligase E3 Component N-Recognin 5

UCH: Carboxy-terminal hydrolase domain

UFM1: ubiquitin-fold modifier 1

UFM1: Ubiquitin-fold modifier 1

UPR: unfolded protein response

UPS: ubiquitin-proteasome system

USP11: Ubiquitin-specific peptidase 11

USP21: Ubiquitin Specific Peptidase 21

UTF1: Undifferentiated Embryonic Cell Transcription Factor 1

WNT8A: Wnt Family Member 8A

WT: Wild-type

WWP2: WW Domain Containing E3 Ubiquitin Protein Ligase 2

Genes and protein terms throughout the thesis adhere to the following guidelines:

- **General**

  Gene/Protein symbol: Capitalized

- **Human:**

  Gene symbol: Capitalized and Italicized

  Protein symbol: Capitalized

- **Mice:**

  Gene symbol: First letter is capitalized, and the symbol is italicized

  Protein symbol: Capitalized

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Resumo

Stress-inducible phosphoprotein 1 (STIP1) é uma proteína evolutivamente conservada, formada por três domínios tetratricopeptídeos repetitivos e dois domínios dipeptídeos ricos em resíduos de aspartato e prolina. A estrutura da STIP1 está intimamente associada à sua multifuncionalidade nas células, sendo encontrada no citoplasma, núcleo e ambiente extracelular. Uma das principais funções da STIP1 é sua atuação como co-chaperona, permitindo a formação de um complexo proteico entre as proteínas de choque-térmico HSP70-HSP90, auxiliando no dobramento e processamento de diversas proteínas clientes dessa maquinaria. O complexo formado pela STIP1 é essencial para a manutenção da homeostase proteica, ou proteostase. A depleção total de STIP1 em camundongos leva a inviabilidade dos embriões, mas apesar do fenótipo elucidado, os mecanismos moleculares exatos que levam a degeneração precoce desses animais não são totalmente conhecidos. Células-tronco embrionárias murinas (CTEm) representam um dos mais eficientes modelos *in vitro* para mimetizar o desenvolvimento embrionário inicial de mamíferos. CTE classificam-se como células-tronco pluripotentes (CTPs), pois possuem a capacidade de se diferenciar em qualquer fenótipo somático adulto sob estímulos adequados, e podem se autorrenovar indefinidamente sem senescência celular. CTPs possuem mecanismos de controle de proteoma avançados, entre eles o aumento na síntese de chaperonas e co-chaperonas, como a STIP1. Muitos fatores cruciais da biologia das CTPs ainda permanecem desconhecidos, e, portanto, compreender as bases moleculares associadas a manutenção da pluripotência é de fundamental importância, tanto para a utilização dessas células em pesquisas científicas, quanto a liberação de seu potencial terapêutico. Nesse sentido, o presente projeto se propõe a lançar luz sobre aspectos associados ao controle de CTPs e seu potencial de diferenciação, proliferação e autorrenovação através dos mecanismos de proteostase. Ainda, buscamos encontrar novas vias moleculares, possivelmente moduladas pela STIP1, associadas ao controle da pluripotência e desenvolvimento inicial de mamíferos.

Palavras-chave: STIP1; proteostase; pluripotência; expressão gênica; células-tronco pluripotentes.

Abstract

Stress-inducible phosphoprotein 1 (STIP1) is an evolutionarily conserved protein formed by three repetitive tetratricopeptide domains and two dipeptide domains rich in aspartate and proline residues. The structure of STIP1 is closely associated with its multifunctionality in cells, being found in the cytoplasm, nucleus, and extracellular environment. One of the main functions of STIP1 is its role as a co-chaperone, allowing the formation of a protein complex between the heat shock proteins HSP70 and HSP90, assisting in the folding and processing of several other proteins, named clients of this molecular machinery. The complex formed by STIP1 is essential for the maintenance of protein homeostasis, or proteostasis. Total depletion of STIP1 in mice leads to embryo degeneration, but despite the elucidated phenotype, the exact molecular mechanisms that lead to early collapse in these animals are unknown. Mouse embryonic stem cells (mESCs) represent one of the most efficient *in vitro* models to mimic early mammalian embryonic development. ESCs are classified as pluripotent stem cells (PSCs), as they can differentiate into any adult somatic phenotype under appropriate stimuli and can self-renew indefinitely without cellular senescence. PSCs have advanced proteome control mechanisms, including increased synthesis of chaperones and co-chaperones, such as STIP1. Many crucial factors in the biology of PSCs remain unknown. Therefore, understanding the molecular bases associated with the maintenance of pluripotency is of fundamental importance, both for the use of these cells in scientific research and the release of their therapeutic potential. Thus, the present project aims to shed light on aspects associated with the control of PSCs and their potential for differentiation, proliferation, and self-renewal through proteostasis mechanisms. Furthermore, we seek to find new molecular pathways, possibly modulated by STIP1, associated with the control of pluripotency and early development of mammals.

Keywords: STIP1; proteostasis; pluripotency; gene expression; pluripotent stem cells.

1. General Introduction

1.1 Proteostasis

Proteostasis refers to the homeostasis of a cell's entire group of proteins. Although the knowledge regarding the need for a regulated proteome is longstanding, the term proteostasis is relatively new and was first introduced in 2008 (Balch *et al.*, 2008). At that time, proteostasis was defined as "controlling the concentration, conformation, binding interactions (quaternary structure), and location of individual proteins making up the proteome by readapting the innate biology of the cell" (Balch *et al.*, 2008). Proteins are the building blocks of cells, which can express from 10,000 to more than 11,000 different proteins, depending on the cell type and status (Kulak *et al.*, 2017). These proteins need to be processed adequately to achieve their function, and this processing spans from their initial synthesis, folding, correct cellular localization, binding with partner proteins, prevention of the formation of potentially pathogenic protein aggregates, refolding and remodelling, and degradation (Hipp *et al.*, 2019). All these pathways and associated proteins comprise the proteostasis network (**Fig. 1A**).

The proteostasis network includes a framework of chaperones, co-chaperones, and associated molecules, including folding and metabolic enzymes, estimated to contain around 2,000 proteins (Klaips *et al.*, 2018; **Fig. 1A**). Additionally, this network encompasses crucial processes such as the unfolded protein response (UPR) (Hetz *et al.*, 2020), protein degradation via the ubiquitin-proteasome system (UPS) and the autophagosomal-lysosomal system (Dikic, 2017), and chaperone-mediated autophagy (CMA) (Bourdenx *et al.*, 2021). These pathways collectively ensure proper cellular functioning in response to various stimuli and contexts, playing a fundamental role in all cell types.

Different processes can lead to proteostasis disruption, from organism ageing to the exposure of cells to pathogens or many sources of stress, including heat shock, genotoxic stress, or oxidative stress (Labbadia and Marimoto, 2015; **Fig. 1B**). Of note, proteostasis is a well-characterized hallmark of ageing and many neurodegenerative diseases, such as Parkinson's disease, Amyotrophic Lateral Sclerosis, and Alzheimer's disease, are associated with proteostasis loss and pathological protein aggregation (Labbadia and Marimoto, 2015; Hipp *et al.*, 2019; Wilson DM 3rd *et al.*, 2023). In addition, other classes of diseases can also be associated with loss of proteostasis, such as type II diabetes and cancer (Chiti and Dobson, 2017).

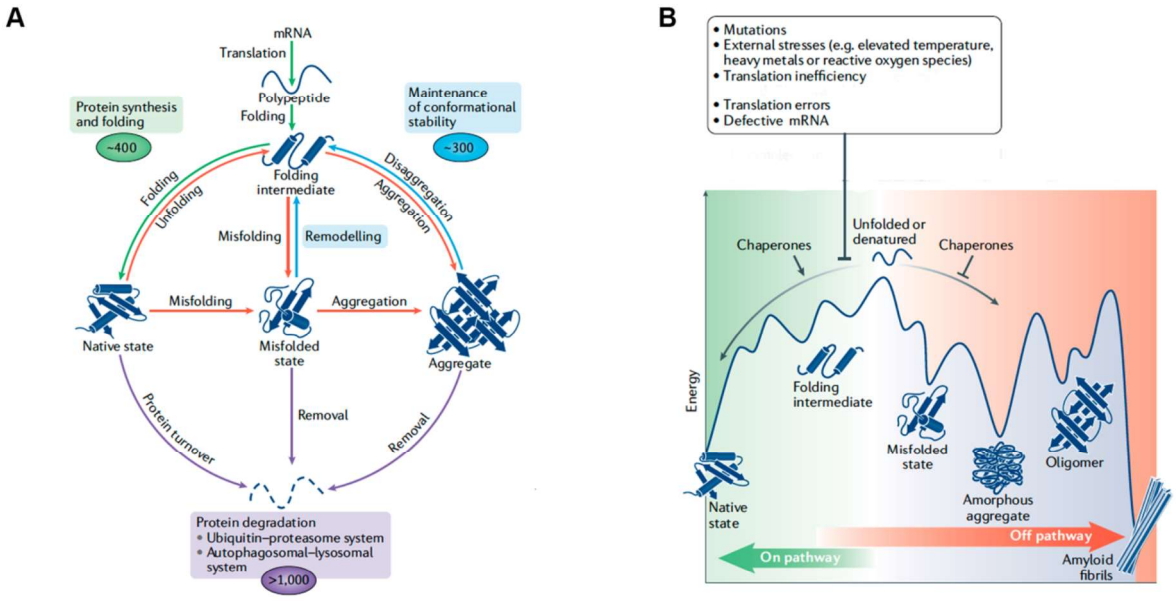


Figure 1: The proteostasis network and its components. (A) The proteostasis network is composed of around 2,000 proteins (in human cells) that are dedicated to protein synthesis and folding of nascent polypeptide chains or folding intermediates (~400 proteins, green); maintenance of conformational stability, avoiding and remodelling pathogenic aggregates and the misfolded state of proteins (~300 proteins, blue); and protein degradation either through the ubiquitin-proteasome system or autophagosomal-lysosomal pathways, controlling the levels of specific proteins and removing aggregates and misfolded proteins (>1,000 proteins, purple). **(B)** Folding intermediates and proteins in their native state (on-pathway processes) are often challenged by different cellular stimuli (i.e., mutations, elevated temperature, reactive oxygen species, translation errors, defective mRNA, etc) and lead the polypeptide chains to off-pathway events, including the formation of misfolded states, amorphous aggregates, pathological oligomers, and amyloid fibrils, that can be thermodynamically stable. Chaperones and co-chaperones work to ensure proper folding while inhibiting the unfolded proteins and aggregates. Adapted from Hipp *et al.*, 2019.

Some proteins, specifically small ones, can refold after their removal from a denaturant environment, even without assisting components (Dobson and Hore, 1998). Although this might suggest that proteins have all the information needed to achieve a functional structure in their sequence, this notion is challenged by bigger and more complex polypeptide chains and the knowledge that the cellular microenvironment is incredibly crowded (Ellis and Minton, 2006). Chaperones and co-chaperones are critical molecules in proteostasis, being highly relevant in the evolution of complex cell types (Hartl *et al.*, 2011). Chaperones can be defined as the molecules that process other proteins, whether assisting in their initial folding, \their binding with other proteins, proteins' proper subcellular localization, or degradation, without being part of their final structure (Hartl, 1996). Proteins assisted by chaperones are frequently named "clients" of that molecular machinery (Hartl *et al.*, 2011; Arhar *et al.*, 2021). There are many chaperone families: small heat shock proteins (sHSPs), HSP40 or J proteins, HSP60, HSP70, HSP90, HSP100, HSP110 and histone chaperones (Hartl *et al.*, 2011; Kim *et al.*, 2013). They form a diverse group of proteins that can share some features, such as being induced by

stress or high conservation through evolutionary history. Among the most prominent molecular chaperone families are the heat shock proteins of 70kDa (HSP70) and 90kDa (HSP90).

HSP70 is a highly conserved chaperone family, from bacteria to humans. It has many cellular functions and is involved in the protein life cycle from initial folding to degradation (Rosenzweig *et al.*, 2019). Although the HSP70 members (13 homologues in humans) have some specificities, such as its sub-cellular localization, they also have overlapping functions (Rosenzweig *et al.*, 2019). In general, HSP70 processes clients through an ATP-dependent cycle. It transiently interacts with exposed hydrophobic regions of nascent polypeptides, promoting proper folding and preventing their aggregation and misfolding events (Liu and Craig, 2016; Rosenzweig *et al.*, 2019; **Fig. 2**). It can also recognize and bind to misfolded or denatured proteins, which can be toxic to the cell if left unchecked (Liu and Craig, 2016; Rosenzweig *et al.*, 2019; Fig. 2). HSP70 has many partners, and its function is assisted by other chaperones, such as those of the HSP40 family and co-chaperones (Hartl *et al.*, 2011). Co-chaperones, in turn, are proteins that assist the function of molecular chaperones, occasionally working as chaperones and possibly sharing some of the chaperone's characteristics (Kim *et al.*, 2013; Hipp *et al.*, 2019). These proteins are fundamental to guarantee good chaperone function within a cell. One of the significant partners of HSP70 and a crucial chaperone for cell function is HSP90 (Genest *et al.*, 2019).

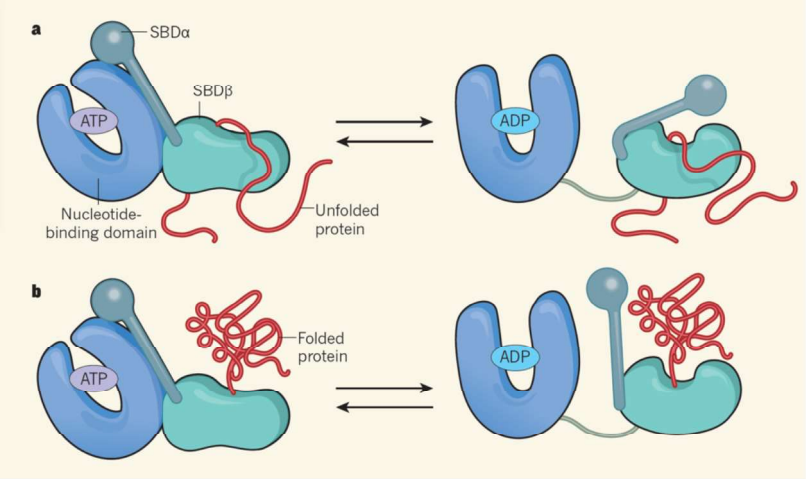


Figure 2: HSP70 functioning in proteostasis. (A) HSP70 family proteins bind to unfolded nascent proteins through its substrate-binding domain (SBDα and β). This structure forms a lid that closes over the unfolded/new polypeptide chain. The lid closing is dependent on ATP binding, which can be hydrolyzed for protein attachment. **(B)** HSP70 can also interact with almost fully folded proteins and prevent misfolding events. Adapted from Liu and Craig, 2016.

Like HSP70, HSP90 is a molecular chaperone crucial for proteostasis of essentially all cell types. HSP90 was also initially identified as a prominent protein induced in response to heat stress (Ritossa, 1996). Subsequent studies revealed the conservation of HSP90 across

diverse organisms, not only in its inducible but constitutively expressed forms, and several different isoforms (Sreedhar *et al.*, 2004; Johnson, 2012). HSP90 is one of the most abundant proteins in the cells. It is responsible for the maturation and regulation of a wide range of clients that include steroid hormone receptors, transcription factors, and protein kinases, among others, establishing HSP90 as a pivotal chaperone for many cellular processes (Zuehlke and Johnson, 2010; Schopf *et al.*, 2017; **Fig. 3**). A complete list of identified clients can be found at https://www.picard.ch/downloads/Hsp90interactors.pdf.

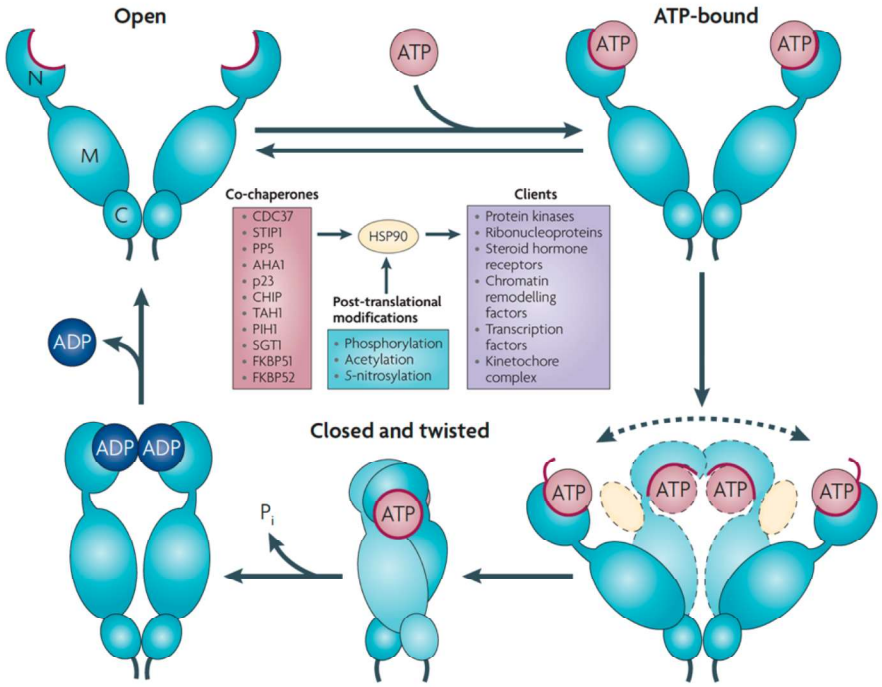


Figure 3: HSP90 molecular machinery and clients. The schematic figure depicts a simplification of the HSP90 machinery cycle. Initially, in its open conformation, the amino-terminal (N) domain of HSP90 binds to ATP, resulting in transient dimerization of its N domains. Subsequent rearrangements that can involve the binding of co-chaperones lead to the 'closed and twisted' conformation that is committed to ATP hydrolysis. Binding with additional co-chaperones can control the hydrolysis rates and ATPase function of HSP90. Upon ATP hydrolysis, the HSP90 goes back to its open conformation for a new cycle. HSP90 is assisted by different co-chaperones (red box), can be post-translationally modified (blue box) and has a wide array of clients that will be delivered to HSP90 to be processed. Adapted from Trepel *et al.*, 2010.

Although HSP70 and HSP90 have isolated roles, the binding of these two chaperones is characterised by functional complementarity (Schopf *et al.*, 2017; Rosenzweig *et al.*, 2019; Gupta *et al.*, 2020; Rutledge *et al.*, 2022). While HSP70 assists in the initial folding and refolding steps, client proteins are transferred to HSP90 by HSP70 for further maturation and functional activation (Lackie *et al.*, 2017). Both proteins have an ATP-dependent chaperone activity that enables them to undergo cycles of ATP hydrolysis-driven conformational changes, which are essential for client protein binding, release, and conformational remodelling. (Trepel *et al.*, 2010; Rosenzweig *et al.*, 2019; **Fig. 2 and 3**). This sequential handover mechanism

ensures client proteins' efficient processing and quality control, contributing to proteostasis. This process is assisted by diverse co-chaperones and accessory proteins. A fundamental co-chaperone in the HSP70-HSP90 machinery is the heat shock organizing protein (HOP), also known as stress-inducible protein 1 (STIP1), which forms a tripartite functional complex with these proteins and secures proper client maturation (Röhl *et al.*, 2015; Lackie *et al.*, 2017; **Fig. 4**).

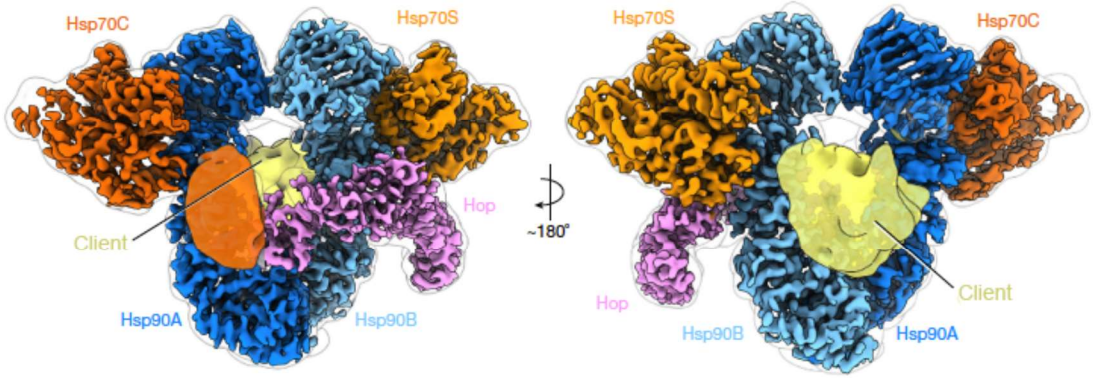


Figure 4: HSP70-STIP1-HSP90 tripartite complex. Cryogenic electron microscopy map of the tripartite complex formed by HSP70, STIP1 and HSP90, with the client protein also present. Front and back are shown. Adapted from Wang *et al.*, 2021.

1.2 Structure and function of STIP1

STIP1, encoded by the *STIP1* gene, was initially described in *Saccharomyces cerevisiae* (yeast) in 1989 (Nicolet and Craig, 1989). This molecule was discovered in the context of stress induction, with a substantial increase in its expression being observed upon heat shock. In addition, the TTC--GAA--TTC--GTA sequence, known as the heat shock element (HSE), was identified at position -241 of its promoter region (Nicolet and Craig, 1989). In yeast, the encoded polypeptide has a structure of 589 amino acids and 66.2kDa (Nicolet and Craig, 1989). Despite its name suggesting that it is inducible by stress (stress-inducible), STIP1 is constitutively expressed in non-stressed cells (Nicolet and Craig, 1989). Subsequently, STIP1 was also described in humans (Honoré *et al.*, 1992) and mice (Blatch *et al.*, 1997; Lässle *et al.*, 1997). The human protein, initially identified as IEF SSP 3521, encodes a molecule of 543 amino acids with a molecular weight of 62.6kDa, 42% identical to yeast STIP1 in its sequence (Honoré *et al.*, 1992). In mice, the protein also has 543 amino acids in its sequence and is significantly similar (97%) to human STIP1. As in mice and humans, STIP1 has also been reported in other groups of eukaryotes, such as *Caenorhabditis elegans* (Gaiser *et al.*, 2009; Song *et al.*, 2009), *Arabidopsis thaliana* (Fernández-Bautista *et al.*, 2018), *Drosophila melanogaster* (Gangaraju *et al.*, 2011; Ambegaokar and Jackson, 2011) and *Trypanosoma cruzi*

(Schmidt *et al.*, 2018), among others, with few structural changes (**Fig. 5**). Therefore, STIP1 is a conserved protein across various organism groups.

The *STIP1* gene, located on chromosome 11 (11q13.1) in humans, consists of 14 exons. Its activation, whether mediated by cellular stress or not, is poorly understood and needs further investigation. It is known that *STIP1* decrease impacts the expression of the transcriptional heat shock transcription factor 1 (HSF1), raising the question if HSF1 could also impact STIP1 expression (Chakraborty and Edkins, 2020). Moreover, *STIP1* is modulated by hypoxia inducible factor 1 alpha (HIF1α) in response to hypoxia (Lee *et al.*, 2013). Recently, the transcription factor cellular tumour antigen p53 (p53) was also associated with *STIP1* regulation. Specifically, Mattison *et al.* (2017) demonstrated that p53 can negatively modulate the *STIP1* promoter activity in tumour cells (Mattison *et al.*, 2017). *STIP1* expression was also shown to be positively regulated by the forkhead box protein c1 (FOXC1) in multipotent arachnoid-pia stem cells by binding directly with its promoter region (Lee *et al.*, 2019). Still, little is known about *STIP1* physiological activation and its implications.

Structurally, STIP1 is mainly formed by tetratricopeptide (TPR) domains and repetitive motifs of 34 amino acids (D'Andrea and Regan, 2003). TPR domains are found in multiple copies in protein sequences, functioning as protein-protein interaction modules that facilitate specific interactions (Allan and Ratajczak, 2010). Briefly, STIP1 consists of three TPR domains, namely TPR1, TPR2A, and TPR2B, each of which is formed from three TPR motifs (Schmid *et al.*, 2012). Additionally, STIP1 is also composed of two dipeptide domains rich in aspartate and proline residues (DP1 and DP2) (Schmid *et al.*, 2012; **Fig. 5**). The structure of STIP1 is closely linked to its multifaceted cellular functions, acting alone or in conjunction with other proteins. STIP1 has been, for many years, classified as a co-chaperone for HSP70 and HSP90, bridging these two molecular chaperones and assisting in client transfer (Röhl *et al.*, 2015). The domains that form STIP1 are intrinsically associated with this function. Mapping the specificity of interaction sites for the HSP70-STIP1-HSP90 complex is challenging due to its rapid formation dynamics. However, research aimed at elucidating these structures suggests that TPR1 is closely linked to HSP70 interaction both *in vivo* and *in vitro*, while TPR2AB is more directly associated with HSP90 binding (Schmid *et al*., 2012; Flom *et al.*, 2007; **Fig. 5**). Although the specific functions of the DP domains are less clear, evidence suggests their importance for STIP1 function in binding the HSP proteins and possible dimerization *in vivo* (Flom *et al.*, 2007), although if indeed STIP1 forms dimers is less clear. Moreover, STIP1 comprises several phosphorylation sites and a nuclear localization signal (NLS) (Blatch *et al.*, 1997; Schwarz *et al*., 2023; **Fig. 5**).

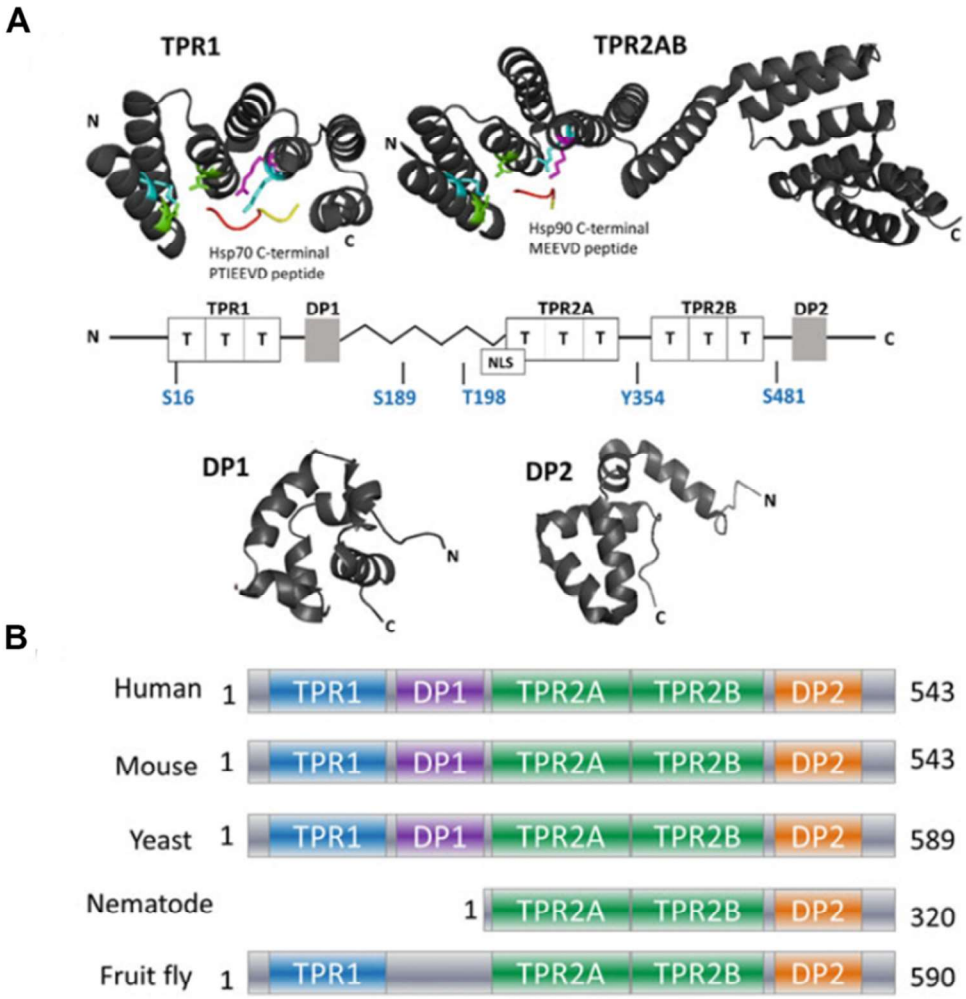


Figure 5: STIP1 structure and conservation in different species. (A) The three-dimensional cartoon illustrates the interaction of STIP1 with HSP70 and HSP90. The TPR1 and TPR2AB, DP1 and DP2 domains of STIP1 are shown. Specifically, TPR1 and TPR2A interact with the C-terminal PTIEEVD motif of HSP70 and the MEEVD motif of HSP90. The phosphorylation residues and the nuclear localization signal (NLS) are identified. **(B)** Organization of STIP1 domains across different species (reproduced from Schwarz *et al.*, 2023).

Its function as a co-chaperone for HSP70-HSP90 primarily occurs in the cytoplasm, where most STIP1 is localized (Schwarz *et al.*, 2023). Although the mechanism of selection and activation of client proteins is not fully known, the HSP70-STIP1-HSP90 machinery works through a process dependent on ATP, with the transfer of client proteins from HSP70 to HSP90, mediated by STIP1 (Flom *et al.*, 2007; Kim *et al.*, 2013; Lackie *et al.*, 2017). In summary, HSP70 recognizes client proteins with exposed hydrophobic regions or unstable folding intermediates. It binds these molecules in its substrate-binding domain (SBD), which has a high affinity for those regions. STIP1 works as a bridge between HSP70 and HSP90, first binding to HSP70, mainly through its TPR1 domains and the C-terminal EEVD motif of HSP70, and subsequently binding to HSP90 (mainly through the TPR2AB domains), facilitating the transfer of client proteins from HSP70 to HSP90 (Flom *et al.*, 2007; Röhl *et al.*, 2015; **Fig. 6**). Once bound to HSP90, client proteins undergo further folding and maturation, stabilizing them

and ensuring their functional competence. While part of this complex, STIP1 inhibits the ATPase activity of HSP90, and its detachment promotes the closing of the HSP90 protein, facilitating client processing (Yamamoto *et al.*, 2014). Post-translational modifications of these chaperones and accessory co-chaperones are critical in regulating thse dynamics and ensuring client proteins' proper folding and activation (Trepel *et al.*, 2010; Rosenzweig *et al.*, 2019; Schwarz *et al.*, 2023; **Fig. 6**).

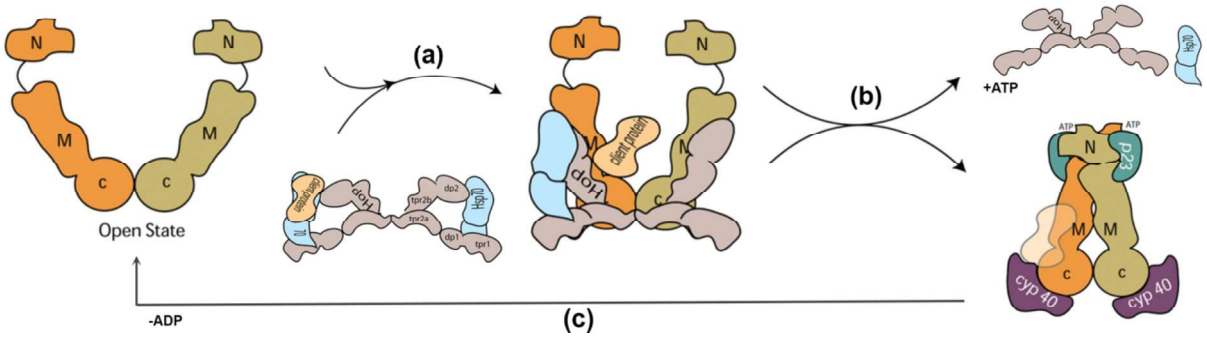


Figure 6: HSP70-STIP1-HSP90 chaperone cycle. Model of the conformational cycle of HSP90. (a) HSP70 and STIP1 attach to HSP90 – in its open conformation -, for client delivery. STIP1 may bind to both monomers of HSP90 through the TPR2B and DP2 domains and prevent ATP hydrolysis. (b) The HSP90–STIP1 complex is weakened upon ATP binding to HSP90, which will trigger conformational changes. Together with the binding of other co-chaperones, STIP1 detaches from the complex. (c) ATP hydrolysis is necessary for client activation and release, with subsequent restoration of HSP90 open state, reinitiating the cycle. Adapted from Onouha *et al.*, 2008.

In addition to its co-chaperone function, there is evidence showing that STIP1 might undertake additional roles within the cell in health and disease. These roles may be directly or indirectly linked to HSP70-HSP90 or completely independent of these proteins. This is supported by the fact that despite being mainly cytoplasmic (Lässle *et al.*, 1997), STIP1 can be found in different sub-cellular locations, including the Golgi apparatus (Honoré *et al.*, 1992), nuclei (Longshaw *et al.*, 2000; Longshaw *et al.*, 2004; Daniel *et al.*, 2008), as the content of extracellular vesicles (Cruz *et al.*, 2018) and in the extracellular environment (Zanata *et al.*, 2002). For instance, STIP1 plays a role in the import of mitochondrial proteins in a process that appears independent of HSP70-HSP90 (Hoseini *et al.*, 2016). This evidence suggests that STIP1 may engage with other critical partners. Moreover, the precise role of STIP1 in the cell nucleus remains incompletely understood. Research suggests a preferential accumulation of STIP1 in the nucleus of astrocytes following UV light irradiation as part of a DNA damage response (Soares *et al.*, 2013). In the extracellular milieu, STIP1 has been found to associate with the cellular prion protein (PrP^{C}), influencing processes such as neuritogenesis, neuroprotection, and astrocyte development (Zanata *et al.*, 2002; Lopes *et al.*, 2005; Arantes *et al.*, 2009). Its evolutionary conservation, multifunctionality, and widespread distribution in various intra- and extracellular localizations underscore a plethora of significant functions that

STIP1 might exert in several pathways associated with the development and homeostasis of organisms.

1.3 Evidence for STIP1's role in the development

Although the evidence is limited, STIP1 has been linked to developmental processes in different organisms. Nematodes (*C. elegans*) lacking STIP1 exhibit reduced lifespan, decreased fertility, and increased lethality under heat stress conditions (Song *et al.*, 2009). Moreover, the depletion of STIP1 in Drosophila ovarian germline nurse cells results in impaired egg hatching and larval development (Karam *et al.*, 2017). However, little is known about STIP1 expression patterns in mammalian embryos and adults. Previous immunohistochemistry work identified that STIP1 starts to be detected from the eighth embryonic day (E8) in mouse embryos, particularly in the neural tube and mesoderm (Hajj *et al.*, 2009). Beraldo and colleagues (2013) described that total depletion of STIP1 (knockout, KO) is lethal in mouse embryos before E10.5 (**Fig. 7**). In fact, few embryos reach this stage in a non-Mendelian ratio, indicating that probably the damages caused by STIP1 KO happen in even earlier developmental stages. Interestingly, these animals showed impaired formation of neural tube and limb buds (Beraldo *et al.*, 2013).

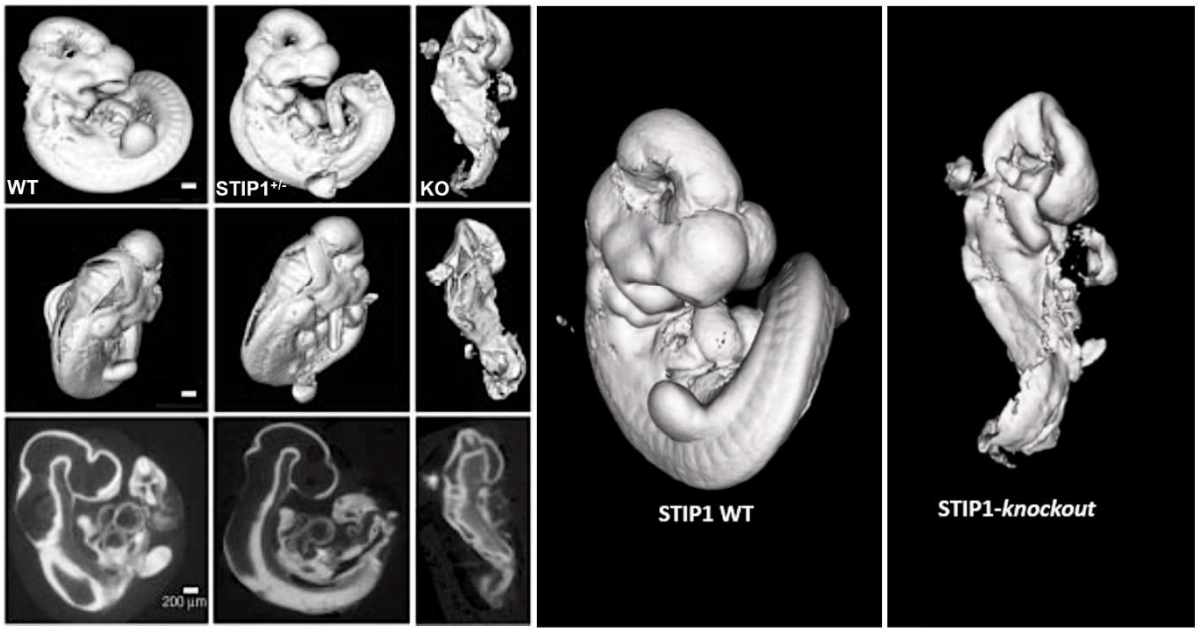


Figure 7: STIP1 knockout is lethal during mouse development. **Left panel:** 3-dimensional images of E10.5 mouse embryos, reconstructed from Micro-CT analysis. The embryos were wild-type (WT), with regular STIP expression; heterozygous knockout ($STIP1^{+/-}$), with 50% expression compared to WT; and the homozygous knockout (KO), with STIP1 complete depletion. Scale bar=200µm. **Right panel:** Same images of WT and KO embryos, but with a better resolution. Adapted from Beraldo *et al.*, 2013.

While the lethal phenotype has been confirmed, the molecular mechanisms underlying the early degeneration of these embryos have not been elucidated. Furthermore, although STIP1 KO is lethal during embryonic development in mice, recent research shows that the KO is not lethal in terminally differentiated human cells (Bhattacharya *et al.*, 2020; Chao *et al.*, 2022), raising the possibility that STIP1 exerts a specific function in cells found during embryonic development. This evidence suggests that STIP1 may play a specific role in early embryonic development or later processes involved in tissue development, such as mesoderm and ectoderm formation. Further studies are needed to precisely understand the molecular pathways regulated directly or indirectly by STIP1 during these processes. In this regard, embryonic stem cells are a fundamental tool for comprehending early embryonic development.

1.4 Pluripotent stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of preimplantation blastocysts. Besides being able to self-renew, these cells are classified as pluripotent stem cells (PSCs) due to their ability to differentiate into all cell types of the three embryonic layers (endoderm, mesoderm, and ectoderm) upon appropriate stimuli (De Los Angeles *et al.*, 2015). ESCs were initially isolated from mice in 1981 by Evans and Kaufman (1981), a feat that earned Evans the Nobel Prize in Physiology or Medicine in 2007. Subsequently, these cells were derived from human blastocysts in 1998 (Thomson *et al.*, 1998). Since their initial derivation, ESCs have represented a significant advancement in scientific research, serving as an *in vitro* model for studying embryogenesis and a valuable tool for genome editing. Moreover, the maintenance of ESCs in culture became much more effective with the discovery of the leukaemia inhibitory factor (LIF), a member of the interleukin 6 superfamilies of cytokines (Ohtsuka *et al.*, 2015). Although LIF is not essential for maintaining ESCs in their undifferentiated state *in vivo*, it plays a crucial role in sustaining pluripotency and self-renewal *in vitro*, particularly for mouse ESCs (mESCs) (Ohtsuka *et al.*, 2015). As research using these tools progressed, other factors were discovered, enabling the *in vitro* maintenance of human ESCs (hESCs) and ESCs derived from other species (Skottman and Hovatta, 2006; Tancos *et al.*, 2012; Solto *et al.*, 2021; Balbasi *et al.*, 2022; Wei et al., 2023).

Pluripotency is a complex cellular state that, although transient *in vivo*, constitutes a critical stage in embryonic development, essential for proper cellular differentiation. It is tightly regulated at various levels, including genetic, epigenetic, post-transcriptional, and metabolic, ensuring precise control over the differentiation process (De Los Angeles *et al.*,

2015). From a transcriptional perspective, among the pivotal molecules sustaining the pluripotency status are the transcription factors POU domain class 5 transcription factor 1 (POU5F1, also known as octamer-binding protein 4 – OCT4), SRY-Box Transcription Factor 2 (SOX2), and homeobox protein NANOG (NANOG) (Loh *et al.*, 2008; Heng *et al.*, 2010; De Los Angeles *et al.*, 2015). These TFs, referred to as the pluripotency core, play a critical role in maintaining PSCs and have been extensively studied over the years (Nichols *et al.*, 1998; Wang *et al.*, 2006; Chambers *et al.*, 2007). A key attribute of this transcription factor core, reflected in its "core" designation, is its ability to bind not only to its own promoters but also to each other, resulting in a highly interconnected self-regulatory mechanism (Yeo and Ng, 2013; **Fig. 8A**). Moreover, these factors are known to regulate a wide genetic network, comprising over 300 targets, which play roles in various aspects of pluripotency, thereby forming an extensive network of transcriptional control (Kim *et al.*, 2008; **Fig. 8B and 8C**).

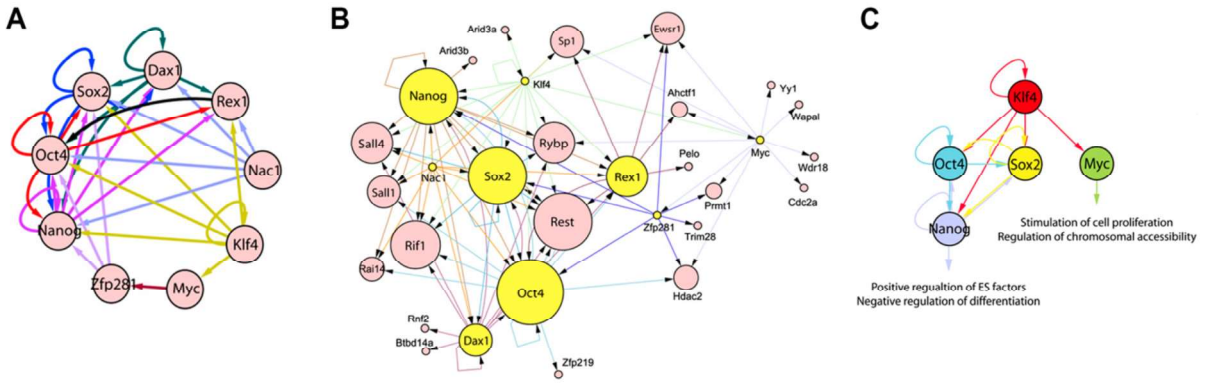


Figure 8: Pluripotency Core and extended network of transcription factors. (A) The transcriptional landscape of pluripotent cells is extremely complex, corresponding to many transcription factors, highlighting the core pluripotency factors OCT4, SOX2 and NANOG, which present autoregulatory mechanisms. Besides them, other factors also harbour the autoregulatory feature, such as KLF4 and DAX1. **(B)** This complex transcriptional landscape comprises an extended network of factors and transcriptional targets. Arrows indicate the direction of transcriptional regulation. Circle size relates to the number of interactions performed by that factor. Some key molecules of (A) are highlighted in yellow. **(C)** The core pluripotency factors are involved in the positive regulation of other pluripotency factors (with more than 300 targets) and with the negative regulation of differentiation. They are also associated with the stimulation of cell proliferation and the regulation of chromosomal accessibility. Adapted from Kim *et al.*, 2008.

Recent research has enabled a parallel comparison between intermediate developmental states and distinct pluripotency statuses, revealing pluripotency as a spectrum rather than a singular state. In other words, stem cells possess different degrees of developmental potential, which can be reflected *in vitro*. Two well-defined stages of pluripotency exist: naïve and primed (Nichols *et al.*, 2009; **Fig. 9**), with intermediate states of pluripotency such as the formative and the rosette stages also described (Smith, 2017; Kinoshita and Smith, 2018; Neagu *et al.*, 2020; **Fig. 9**). These states are characterized by specific molecular and functional

characteristics (Kinoshita and Smith, 2018), adding complexity to our understanding of pluripotency models and shedding light on discrete cell-fate changes throughout development.

Despite their enormous potential, ESCs are restricted by ethical constraints, primarily due to their derivation, which necessarily involves the interruption of embryo development. Considering human cell lines, these ethical restrictions are incredibly complex, and laws vary according to each country (Lo and Parham, 2009). A new scientific advance resolved most of these ethical implications in the following years of ESC derivation. Induced pluripotent stem cells (iPSCs) were derived from mice in 2006 by Yamanaka's group (Takahashi and Yamanaka, 2006), and soon after, they also reported the derivation of human iPSCs (hiPSCs, Takahashi *et al.*, 2007). These researchers described a method for reprogramming adult cells - in that case, fibroblasts - so they would present very similar characteristics to ESCs (Takahashi *et al.*, 2007). This was precisely accomplished by the induction of four genes: OCT4, SOX2, KLF transcription factor 4 (KLF4), and MYC proto-oncogene (c-MYC), later known as Yamanaka factors (Liu *et al.*, 2008). iPSCs have revolutionized the field of stem cell research, providing a ground-breaking approach to studying human development, disease modelling, drug discovery, and regenerative medicine (Shi *et al.*, 2016; Karagiannis *et al.*, 2019; Mertens *et al.*, 2018; Bose *et al.*, 2022). This accomplishment was prized the Nobel in Physiology or Medicine in 2012. With the advancement of research on this technology, various cell types and reprogramming factors have been explored, providing alternatives for many ethical issues surrounding stem cell research (Takahashi and Yamanaka, 2016; Shelby *et al.*, 2022).

ESCs and iPSCs have become indispensable tools for scientific research, with numerous studies aiming to elucidate the extent of similarities and differences between these two models (Takahashi and Yamanaka, 2006; Chin *et al.*, 2009; Halevy and Urbach, 2014). Both cell types are governed by a robust transcriptional network involving core transcription factors and other levels of control that must be finely regulated to ensure the pluripotent phenotype (Liang and Zhang, 2012). Although iPSCs share many characteristics with ESCs, they possess intrinsic differences attributed to the terminally differentiated cells from which they originate (Efrat, 202; Scesa *et al.*, 2021). These differences warrant further investigation to fully understand the biology of these cells and ensure their safe and effective use in scientific research and potential therapeutic applications.

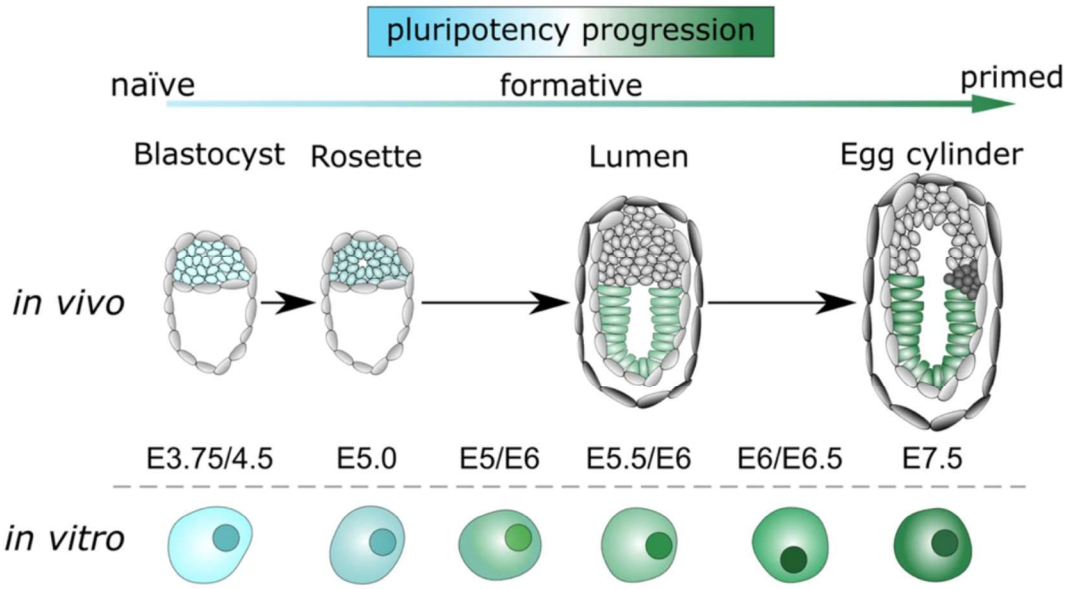


Figure 9: Pluripotency is a spectrum. This schematic figure depicts the pluripotency spectrum, from naïve to intermediate/formative to primed pluripotency states. These states mirror the developmental phases of the embryo (blastocyst, rosette, lumen, and egg cylinder) from which pluripotent cells can be derived and subsequentially maintained *in vitro*. Adapted from Furlan *et al.*, 2023.

1.5 Proteostasis can regulate pluripotency and development

While the regulation of pluripotency has been thoroughly investigated at the genetic and epigenetic levels, mechanisms involving post-transcriptional control, particularly those related to proteostasis, remain poorly understood. A search for the terms "proteostasis" and "pluripotency" on the PubMed platform returns only 94 results (**Fig. 10A**, accessed in March 2024), showing the scarcity of specialized literature exploring this aspect of PSCs' biology. Of note, those papers encompass all the mechanisms associated with proteostasis, not just those related to chaperones and co-chaperones' function.

The literature suggests that the continuous proliferation of PSCs, in addition to their unique characteristics, requires high rates of translation (Vilchez *et al.*, 2014; García-Prat *et al.*, 2017). This heightened capacity for protein synthesis is closely linked to the necessity for robust proteome quality control mechanisms, emphasizing the critical role of proteostasis in maintaining pluripotency (Lee *et al.*, 2017). Stem cell viability crucially relies on maintaining protein homeostasis, enabling the continuous adaptation of the cellular proteome to both extrinsic and intrinsic variations (Lee *et al.*, 2017). Additionally, the ability of stem cells to perceive and respond to changing conditions and stress is vital for cell growth, development, and organismal viability (Llamas *et al.*, 2020). Consequently, it is proposed that these cells exhibit elevated rates of chaperone and co-chaperone synthesis and activity, as well as heightened activity of the UPS (Noormohammadi *et al.*, 2018; Llamas *et al.*, 2020; **Fig. 10B**).

Although some evidence points to these hypotheses, such as increased proteasome activity in PSCs (Vilchez *et al.*, 2012), the research field lacks robust evidence regarding the function of proteostasis, chaperone and co-chaperone proteins in the biology of these cells.

Given the scarcity of evidence in the literature on the importance of proteostasis in maintaining pluripotency and, consequently, in the early stages of embryonic development, in this project, we proposed to elucidate aspects related to the regulation of PSCs and their potential for differentiation, proliferation, and self-renewal through proteostasis mechanisms, particularly through the function of STIP1. Furthermore, we aimed to identify new molecular pathways that STIP1 possibly modulates, associated with the control of pluripotency and early mammalian development.

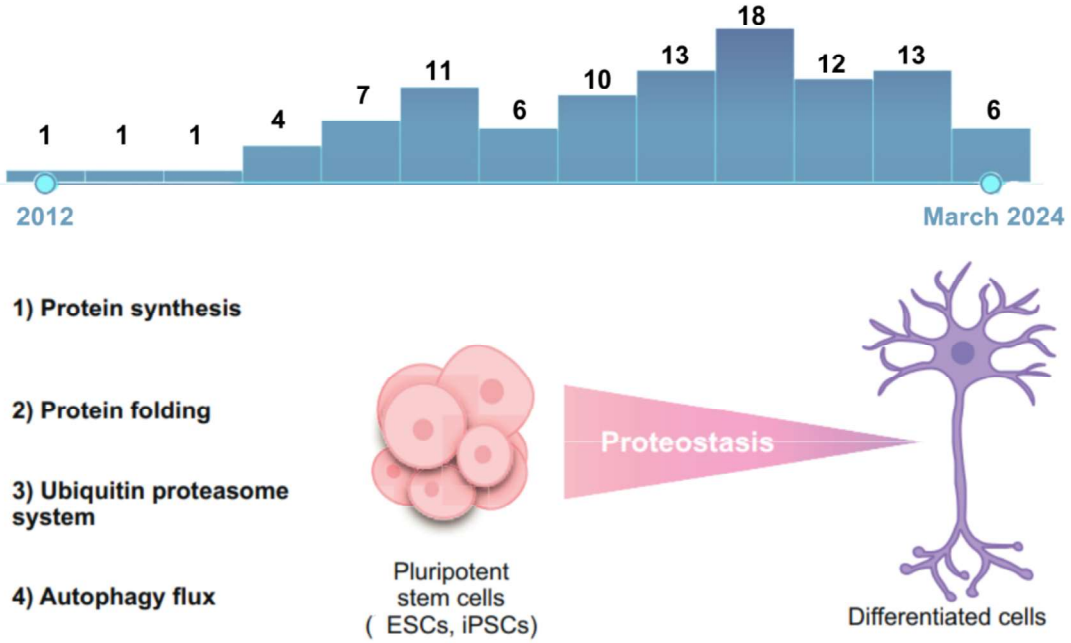


Figure 10: Proteostasis in pluripotency. Despite being an important topic, the control of pluripotency through proteostasis is a relatively under-explored theme in the specialized literature. Upper: Bar chart shows results for searching the terms "(proteostasis) and (pluripotency)" in PubMed (Access in March 2024). Only 94 articles were found. Lower: Pluripotent stem cells, including ESCs and iPSCs, present enhanced mechanisms of protein synthesis, protein folding, ubiquitin-proteasome system, and autophagy relative to differentiated cells. Adapted from Llamas *et al.*, 2020.

2. Aims

2.1 General

Investigate the function of STIP1 in maintaining the proteostasis network and controlling pluripotency in PSCs.

2.2 Specifics

2.2.1 **To characterize the WT, STIP1$^{+/-}$, STIP1$^{\Delta TPR1}$, and STIP1^{TgA} mESC lines for the expression of pluripotency, proliferation, apoptosis, and DNA damage markers.** Approach: Characterize mESCs derived previously by our group from the ICM of blastocysts of genetically modified mice for the *STIP1* gene, therefore expressing different levels of STIP1 (namely wild-type, WT; STIP1$^{+/-}$, haploinsufficiency model comprising a heterozygous knockout; STIP1$^{\Delta TPR1}$, expressing a truncated isoform; and STIP1^{TgA}, overexpressing model), for the expression of pluripotency markers (alkaline phosphatase; pluripotency core OCT4, SOX2 and NANOG and other additional factors), at the protein and gene expression levels. Furthermore, we will quantitatively analyse the proliferation of the aforementioned cell lines and the expression of apoptosis (cleaved caspase-3) and DNA damage (phosphorylated H2A.X) markers.

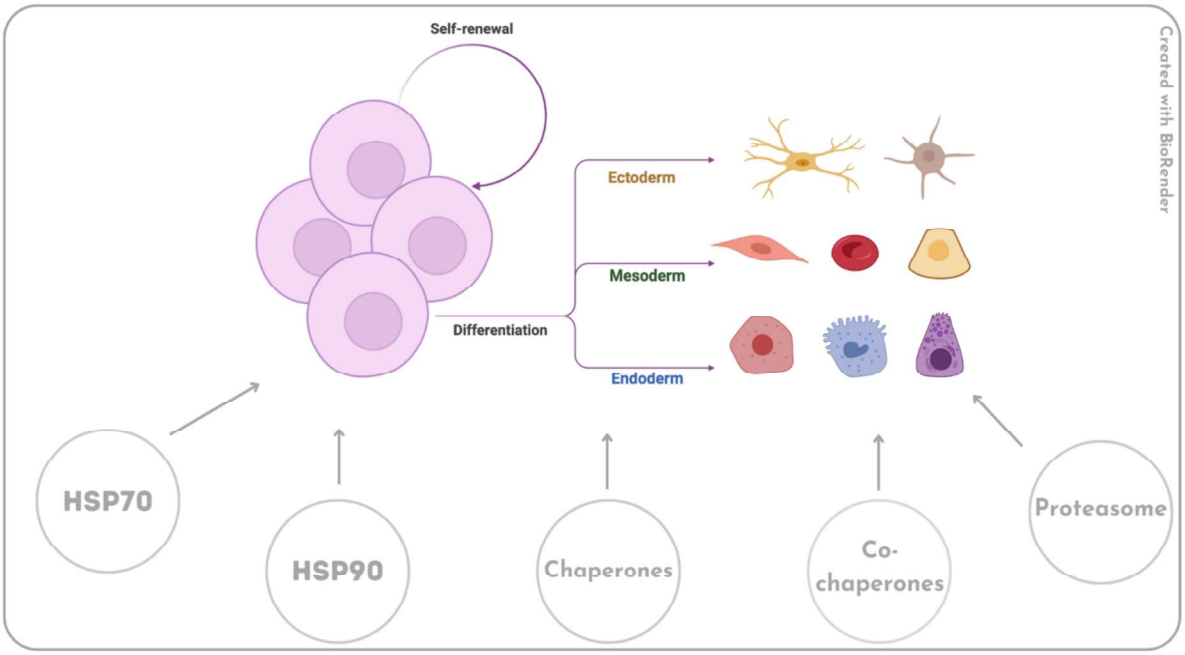
2.2.2 **To determine the impacts of variable STIP1 expression on the regulation of PSCs resilience.** Approach: Using the mESCs WT, STIP1$^{+/-}$, STIP1$^{\Delta TPR1}$, and STIP1^{TgA}, we seek to elucidate the importance of STIP1 in regulating the resilience of PSCs. The sensitivity of the cells will be tested through functional assays for oxygen-glucose deprivation (hypoxia), exposure to factors that stimulate increased apoptosis (staurosporine), heat shock (activation of the heat shock response), and cellular stresses, such as increased oxidative stress (exposure to H_2O_2 for example).

2.2.3 **To establish whether the different phenotypes observed with variable expression of STIP1 can be rescued with the induction of wild-type STIP1 expression.** Approach: Rescue assays will be carried out with the aim of understanding if the phenotypes observed in mESCs are due to the variation in STIP1 levels, further characterizing the molecular basis of those phenotypes. From the induction of wild-type STIP1 expression in STI1$^{+/-}$ and STIP1$^{\Delta TPR1}$ mESCs, using plasmids already available in the laboratory, we will investigate the expression

of OCT4, SOX2 and NANOG and the possible rescue of the pluripotent phenotype. Alternatively, we will use stable expression induction techniques using lentivirus or AAV.

2.2.4 **To investigate the effects of variable STIP1 expression on phenotypic commitment decisions of PSCs.** Approach: To investigate whether changes in STIP1 levels or the expression of a truncated isoform of STIP1 ($STI1^{\Delta TPR1}$) can direct mESCs to any differentiation bias. Using the cultivation of monolayer cells and the formation of embryoid bodies, the rate of differentiation and the expression of specific markers for the three germ layers (endoderm, mesoderm, and ectoderm) will be evaluated in all cell lines.

2.2.5 **To examine the importance of STIP1 in the proteostasis machinery of hiPSCs.** Approach: To determine whether the phenotypes, signalling pathways and other factors relevant to the biology of PSCs with differential expression of STIP1, previously identified in the mESCs study, are also present in human iPSCs. This branch of the project was developed in a collaborative study during a Research Internship Abroad (RIA/BEPE) at the laboratory of Dr. David Vilchez (Cluster for Excellence in Aging Research – CECAD, University of Cologne, Cologne - Germany), a researcher with extensive experience in the use of human iPSCs, protein homeostasis and its importance for the maintenance of pluripotency.



3. Chapter I

The role of chaperones, co-chaperones and the proteostasis network in pluripotency maintenance

Literature review article titled ***Chaperones and Beyond as Key Players in Pluripotency Maintenance*** published in Frontiers in Cell and Developmental Biology on August 2, 2019

This chapter presents a literature review article focusing on the functions of various classes of chaperones and co-chaperones, as well as the mechanisms of the UPS, in preserving pluripotency in stem cells. The inspiration for this review stemmed from the observation that no existing review comprehensively synthesized both classic and recent research on the functions of these systems in maintaining the stemness and biology of PSCs. The review delves into the roles of the UPS, histone chaperones, molecular chaperones, and associated proteins in diverse cellular processes responsible for preserving the pluripotent phenotype in mESCs and hESCs, as well as iPSCs.

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Chaperones and Beyond as Key Players in Pluripotency Maintenance

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Pluripotency is orchestrated by distinct players and chaperones and their partners have emerged as pivotal molecules in proteostasis control to maintain stemness. The proteostasis network consists of diverse interconnected pathways that function dynamically according to the needs of the cell to quality control and maintain protein homeostasis. The proteostasis machinery of pluripotent stem cells (PSCs) is finely adjusted in response to distinct stimuli during cell fate commitment to determine successful organism development. Growing evidence has shown different classes of chaperones regulating crucial cellular processes in PSCs. Histones chaperones promote proper nucleosome assembly and modulate the epigenetic regulation of factors involved in PSCs' rapid turnover from pluripotency to differentiation. The life cycle of pluripotency proteins from synthesis and folding, transport and degradation is finely regulated by chaperones and co-factors either to maintain the stemness status or to cell fate commitment. Here, we summarize current knowledge of the chaperone network that govern stemness and present the versatile role of chaperones in stem cells resilience. Elucidation of the intricate regulation of pluripotency, dissecting in detail molecular determinants and drivers, is fundamental to understanding the properties of stem cells in order to provide a reliable foundation for biomedical research and regenerative medicine.

Keywords: pluripotency, chaperone, stem cells, histone, stemness

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INTRODUCTION

Pluripotency is an important and unique feature attributed to specific types of cells, and can be defined as the ability of cells to replicate indefinitely in the absence of senescence (self-renewal) while retaining the differentiation potential, or the ability to differentiate into all cells of an organism (Martello and Smith, 2014). Embryonic stem cells (ESCs) are classified as pluripotent stem cells (PSCs), and represent great possibilities for research and cell therapy. ESCs can be obtained from the inner cell mass (ICM) of preimplantation blastocysts. The establishment of cultures of ESCs *in vitro* (Evans and Kaufman, 1981; Martin, 1981; Martello and Smith, 2014) brought about unquestionable advances in scientific research, as the starting point for several works that sought to explore the molecular mechanisms that maintain pluripotency. In 2006, a state of ESC-like, achieved from the reprogramming of differentiated adult cells was described, referred to as induced pluripotent stem cells (iPSCs). Reprogramming of the cells was possible through the induction of specific transcription factors (TFs), OCT4, SOX2, c-MYC, and KLF4 (Takahashi and Yamanaka, 2006). OCT4, NANOG, and SOX2 are considered key factors for the maintenance of PSCs *in vivo* and *in vitro*, forming a pluripotency core that, with additional TF and cofactors, regulates pluripotency in an expanded transcriptional network (Wang et al., 2006; Kim et al., 2008).

Although many studies have been conducted to understand the exact mechanisms by which the TFs and additional factors regulate pluripotency, much remains to be elucidated. Studies are still being conducted to understand TFs individual and integrated functioning. By unmasking pluripotency mechanisms, it will be possible to use PSCs more safely and harness their therapeutic potential, also serving as a model to understand early development, important cellular processes and diseases. Besides transcriptional regulation, other mechanisms are being recently discussed as relevant to understanding the maintenance of pluripotency in stem cells, such as chromatin conformation and proteome quality control assisted by molecular chaperones. In this review we discuss aspects of PSCs maintenance, such as TFs regulation and chromatin conformation in PSCs, as well as the relationship of chaperones, co-chaperones and ubiquitin-proteasome system (UPS) with the control of TF levels and pluripotency in PSCs. A comprehensive and integrated understanding of the events – from transcription, translation to post-translational processes – that govern pluripotency is needed to answer questions that remain unanswered in the field of PSCs.

PLURIPOTENCY MAINTENANCE MECHANISMS

Pluripotent stem cells are regulated by a series of interconnected cellular processes that pass through the transcription, translation, and final destination of proteins through different post-translational modifications. The state of chromatin conformation is important for the exposure or concealment of regulatory regions in DNA. Regulatory regions are possible targets of several TFs, that will associate in a specific way in the DNA molecule and regulate the transcription of several genes.

Several studies described the essentiality of the TFs OCT4, SOX2, and NANOG to pluripotency. These TFs form an interconnected self-regulating core, cooperatively associating with their own promoters and co-occupying more than 300 targets in an integrated manner, finely regulating their own and also their targets expression, repressing differentiation genes and activating pluripotency (Boyer et al., 2005; Wang et al., 2006). Other TFs (referred hereafter as expanded core) associated to the main pluripotency core were described, such as STAT3, SMAD1, DAX1, REX1, ZPF281, among others (Kim et al., 2008; Bharathan et al., 2017; Collier et al., 2017; Trusler et al., 2018). In 2006, the publication of Takahashi and Yamanaka's (2006) landmark work, describing the acquiring of PSCs from the induction of four major TFs – OCT4, SOX2, c-MYC, and KLF4 – profoundly impacted stem cell research, allowing the field of development to dispose of a new and effective tool for pluripotency studies.

Although pluripotency is controlled by the expression of a network of TFs, the levels of this expression must be highly regulated in order to maintain the pluripotent state. PSCs balance between self-renewal and differentiation potential. Some researches (briefly reviewed in Torres-Padilla and Chambers, 2014), have already shown that the levels of TFs associated with pluripotency may vary in ESCs. Even in cells of the same colony, the expression of certain factors can be heterogeneous and transient. In addition to transcriptional regulation, the levels of TFs can be modulated according to translational and degradation rates and post-translational modifications.

In order to maintain PSCs undifferentiated, a cytokine member of the IL6 superfamily, named leukemia inhibitory factor (LIF), is used in cell culture (Nicola and Babon, 2015). Briefly, in the signal transduction cascade, LIF couples with gp130 receptors and activates JAKs in the cell interior which, in turn, will activate STAT3 through phosphorylation (Huang et al., 2017). Although LIF is not essential for the maintenance of these cells *in vivo* (Stewart et al., 1992), and is not solely responsible for the maintenance of pluripotency and self-renewal *in vitro*, it is an important tool in the culture of ESCs and iPSCs. Later in this review we will discuss how STAT3 relates to an important protein complex of chaperones and co-chaperones, acting in the maintenance of pluripotency in stem cells *in vitro*.

Interestingly, the existence of different statuses of pluripotency has been reported, naïve and primed, with great variety in transcriptional and epigenetic profile (reviewed in Nichols and Smith, 2009; Hackett and Azim Surani, 2014). The primed state, as the name suggests, is a state more prone to differentiation when compared to the naïve state (Marks et al., 2012), although in both states the cells remain expressing pluripotency core TFs. In addition to these classically established states, the existence of other levels for pluripotency has recently been hypothesized. As recently proposed by Smith, pluripotency can be seen as a progression through very early different developmental stages. The author emphasizes the need for a formative pluripotency state between the naïve and primed, in which cells acquire abilities to change their genomic and epigenetic profile to proceed in the course of cell-fate commitment (Smith, 2017). While some authors defend great transcriptional heterogeneity between primed and naïve states (Marks et al., 2012), other discuss that, although the two populations have their specific transcriptional signatures, this heterogeneity is expressed in low levels between the states (Messmer et al., 2019). Much still needs to be studied in order to establish a response to these controversies. Studies exploring these states *in vivo* may contribute to the understanding of their existence as part of the development of organisms, or as artifacts of cell culture.

Pluripotent stem cells require elevated protein synthesis for continuous replication and thus, enhanced mechanisms of proteome quality control like elevated chaperone and proteasome activities is essential to avoid senescence and maintain stemness. The viability of stem cells critically depends on the ability to maintain protein homeostasis to adapt continuously the cellular proteome to extrinsic and intrinsic variations. The capacity of stem cells to sense and respond to changing conditions and stress is critical for normal cell growth, development and organism viability. The complexity of the proteome requires interconnected quality-control processes to meet the dynamic needs of the cell. The protein homeostasis (proteostasis) network (PN) ensures the balance of the proteome by coordinating protein synthesis, folding and conformational maintenance; and protein degradation. PN is achieved by an orchestrated system of proteins, including molecular chaperones and their regulators, which help proteins to reach its functionally active conformation,

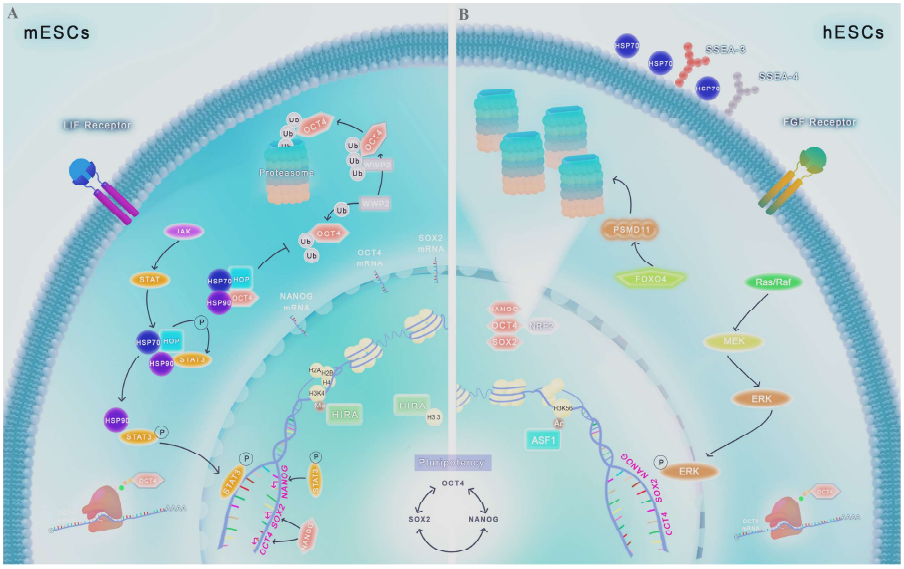

FIGURE 1 | Chaperome regulation and proteostasis network in ESCs. Scheme shows molecular pathways ranging from gene transcription to protein degradation involved in pluripotency control. The interconnected self-regulating nuclear core formed by OCT4, SOX2, and NANOG is essential for the maintenance of stemness. **(A)** In mESCs, HIRA is abundantly associated with promoter regions of developmentally regulated genes, being responsible for H3.3 deposition and enrichment, co-localizing with the transcriptional active form of methylated H3K4. Chaperone protein HSP90 and its partner HOP are engaged in key intracellular signaling pathways in PSCs, including LIF/JAK/STAT3. HSP90-HOP complex participates actively in the phosphorylation and translocation of STAT3 to the nucleus, leading to the transcription of pluripotency core factors. HSPs complexes can also prevent OCT4 degradation by proteasome. Proteasome-related proteins, such as WWP2, acting as E3 ligases or by other mechanisms, lead to TFs degradation by UPS, controlling its levels and maintaining proteostasis balance in these cells. **(B)** In hESCs, FGF2, used to culture these cells, activate the signaling cascade mediated by Ras/MEK/ERK and p-ERK translocation to the nucleus, favoring the expression of pluripotency genes. Acetylation of H3K56 by ASF1 regulates de expression of pluripotency genes. Unlike differentiated cells, HSP70 is present in the cell surface of hESCs, colocalizing with known pluripotency markers such as SSEA3 and SSEA4. Upregulation of the protein FOXO4 leads to the increase of the 19S proteasome subunit PSMD11, resulting in more functional proteasome subunits formed and increased activity of the UPS. The TF NRF2 upregulation is also associated with the increase in functional proteasome subunits, and also is associated with expression of the pluripotency TFs OCT4, SOX2, and NANOG.

without being part of its final structure. In addition, the UPS exerts a post-transcriptional control on the levels of proteins, such as TFs, which is important to pluripotency maintenance (**Figures 1**, **2**; Okita and Nakayama, 2012).

Considering the fine mechanics of chromatin conformation control, the importance of PN for the maintenance of cellular functions, both in health and in diseases, the increased expression of PN elements in PSCs, as well as an increased activity of PN in these cells, many studies have been conducted to understand the control of pluripotency from the perspective of these events. **Tables 1**, **2** summarize different molecules, addressed in this review, involved in the pluripotency control.

TFs CORE REGULATION BY UBIQUITIN-PROTEASOME SYSTEM

Regardless of the mediation of chaperones, an important fraction of polypeptides usually exhibit errors in folding or refolding (Schubert et al., 2000); consequently, they are identified and disposed by proteolytic degradation, to avoid accumulation of potentially toxic aggregate species. One major protein degradation pathway is the UPS. The UPS performs coordinated activities of enzymes that conjugate the polypeptide co-factor, ubiquitin, to proteins and tags them for degradation by an ATP-dependent process that involves three enzymes, E1 (Ub-activating enzyme), E2s (Ub-carrier or conjugating proteins) and the key E3-ligases (Ub-protein ligase) (Schubert et al., 2000; Lecker et al., 2006). The labeled proteins are identified by the 26S proteasome, which degrades them to small peptides (Lecker et al., 2006).

The protein levels of pluripotency TFs must be finely regulated for the maintenance of PSCs specific properties. It has been shown that both downregulation and upregulation beyond the required levels of some TFs, such as OCT4 (Rodriguez et al., 2008; Zafarana et al., 2009), leads to differentiation in specific tissues or impairment in stem cell identity. The UPS is one of

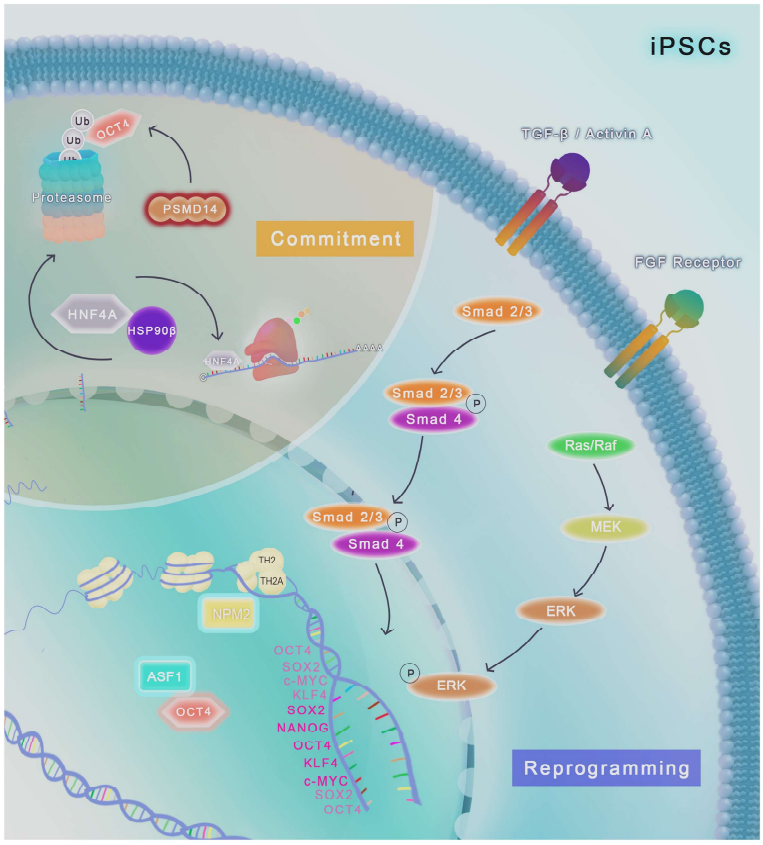

FIGURE 2 | Chaperome regulation and proteostasis network in human iPSCs. TGF-β/Activin A and FGF2/Ras/MEK/ERK pathways are required for the maintenance of iPSCs in culture conditions. The histone chaperone NPM2 binds to the histone variants TH2A and TH2 and improve the reprogramming of human fibroblast into iPSCs modulated by OCT4, SOX2, KLF4 and c-MYC, generating more naïve human iPSCs compared to factors induction alone. ASF1a histone chaperone upregulation, together with OCT4, also has an important role in reprogramming of human fibroblast. Cell fate commitment (highlighted in light brown) involves the induction of different specific pathways that can lead to differentiation into various cell types. The molecular chaperone HSP90β physically binds to HNF4A and control the protein turnover of these client, modulating differentiation of iPSCs to endoderm-derived hepatic progenitor cells. Downregulation (represented as a red glow around the molecule) of the proteasome-related protein PSMD14, a 26S proteasome subunit, impairs the deubiquitylation of OCT4, leading to its degradation in the proteasome and impairment of pluripotency.

the main post-translational mechanisms for regulating the levels of these proteins. The selectivity of proteins and aggregations for ubiquitin system presents a relevant participation of chaperones and co-chaperones, more specifically from the HSP70 family, for example HSC70 and the co-chaperone BAG3 (Arndt et al., 2010). In this section, we highlight classical and recent findings that explore the role of UPS in the control of TFs levels, essential for PSCs maintenance.

Investigating the total profile of ubiquitinated proteins in mESCs and iPSCs, Buckley et al. (2012) revealed that NANOG and OCT4 are regularly ubiquitinated, with the levels of this modification varying throughout the self-renewal process, a phenomenon that is not observed in differentiated cells (Buckley et al., 2012). The work demonstrates how these TFs are finely regulated for the maintenance of pluripotency properties in these cells, and suggests the great importance of UPS for self-renewal. It is known that elevated proteasome activity is somehow essential not only for the control of TFs levels, but also for expression of genes associated with pluripotency, cell proliferation and cell cycle progression. Defects in the proteasome leads to malfunction

TABLE 1 | Major classes of histone chaperones and their function in stemness of different PSCs models.

Chaperone		Function in PSCs biology	References
HIRA		Highly expressed in the promoters of developmentally regulated genes in mESCs	Goldberg et al., 2010
		Differentiation of mESCs in hemogenic endothelium	Banaszynski et al., 2013; Scambler et al., 2015
		Pluripotency maintenance of hESCs, promoting isocitrate dehydrogenase genes (IDHs) transcription	Zhu et al., 2017
		Developmental reprogramming – deposition of paternal core histone and reactivation of maternal genome in mice	Lin et al., 2014
DAXX/ATX		Telomeric deposition (immortalization) in mESCs	Elsässer et al., 2015
ASF1		Differentiation during murine early embryogenesis and gonad development	Messiaen et al., 2016
		Pluripotency maintenance in hESCs	Gonzalez-Muñoz et al., 2014
		Pluripotency maintenance in mESCs	Tan et al., 2013
		Reprogramming of human fibroblasts into iPSCs	Gonzalez-Muñoz et al., 2014
CAF-1		Early developmental arrest and early gastrulation of mESCs	Filipescu et al., 2013; Akiyama et al., 2011; Houlard et al., 2006; Hatanaka et al., 2015
		Reprogramming of mouse fibroblasts into iPSCs	Cheloufi et al., 2015
		Pluripotency maintenance during blastomeric stage in mice	Yankulov, 2015
FACT		Proliferation and neural differentiation of mESCs	Mylonas and Tessarz, 2018
		Associates with OCT4 and regulates mESCs pluripotency Survival during early blastocyst stage of mESCs	Cao et al., 2003; Ding et al., 2012; Gaspar-Maia et al., 2009; Pardo et al., 2010
		Reprogramming into iPSCs	Shakya et al., 2015; Shen et al., 2018
HMGA2		mESCs specific DNA repair mechanism.	Yu et al., 2014
NPM2		Reprogramming of human fibroblast into iPSCs; Improvement of murine cells reprogramming using only KLF4 and OCT4	Fernández-Rivero et al., 2016; Shinagawa et al., 2014
NPM3		Proliferation of mESCs	Motoi et al., 2008
SPT6		Pluripotency maintenance of mESCs	Robert, 2017
SET	SETα	Proliferation of hESCs	Edupuganti et al., 2017
	SETβSETP	Differentiation of hESCs	

of all of the above processes, including a G2/M arrest in hESCs and iPSCs (Jang et al., 2014). Several research groups have been exploring the specific effects of UPS-associated proteins on the regulation of TFs in PSCs.

Upon the downregulation of PSMD14, a 26S proteasome non-ATPase subunit, the loss of OCT4 expression is observed, which is linked to an apparent dysfunction of the deubiquitinating enzymatic activity of PSMD14 (**Figure 2**; Buckley et al., 2012). Further, although the modulation of the E3 ligase protein FBXW7 has no direct effects on OCT4, NANOG and SOX2 expression, it has a negative effect on the protein stability of c-MYC, an important factor linked to the differentiation potential in PSCs (Buckley et al., 2012). NRF2 is a TF whose activation leads to increased levels of several proteasome subunits. NRF2 activity is increased in hESCs (**Figure 1B**) and iPSCs and loss of this activity results in reduced levels of OCT4, SOX2, and NANOG and also impairs proliferation, indicating a role of this protein in self-renewal (**Figure 1B**; Jang et al., 2014). Additionally, NRF2 colocalizes with the mentioned core TFs (e.g., OCT4, SOX2, NANOG) during differentiation, and its activation by pharmacological or molecular techniques prevents their degradation during differentiation process in hESCs (Jang et al., 2014).

The proteasome activity is enhanced in hESCs and iPSCs, with a loss of activity being observed as the cells differentiate, with a concomitant increase in differentiation factors, such as PAX6, as well as a decrease in the expression of pluripotency TFs (Vilchez et al., 2012). Furthermore, the transcription factor FOXO4 has been shown to be an important modulator of proteasome activity in hESCs, since it regulates the expression PSMD11, a 19S proteasome subunit. The increased expression of PSMD11 is sufficient to increase the number of functional proteasome complexes formed, increasing proteasomal activity (**Figure 1B**; Vilchez et al., 2012). Interestingly, PSMD11 levels are somehow related with other pluripotency proteins. Decreased levels of L1TD1, an RNA binding protein, correlates with decreased levels of PSMD11 (Emani et al., 2015). Pharmacological inhibition of proteasome activity leads to decrease in L1TD1 and SOX2 in hESCs (Emani et al., 2015). However, the exact mechanisms by which these molecules interact with each other, and how they interact with OCT4, SOX2, NANOG, and other pluripotency TFs still needs to be better studied.

The COP9 signalosome (CSN) is an important protein complex that prevents protein degradation, and was previously implicated in pluripotency maintenance (Chia et al., 2010). Experiments using knockdown (KD) mESCs for COPS2 protein, a specific subunit of the CSN, showed that COPS2 regulates protein stability of NANOG by its independent and direct interaction with the α-helixes 2 and 3 of NANOG's homeobox domain, preventing its degradation by the proteasome

TABLE 2 | List of chaperones and proteasome-related proteins and their function associated to protein homeostasis and pluripotency control in different PSCs models.

Proteins and families		Function in PSCs biology	References
Chaperome	HSP90	Pluripotency maintenance and mesoderm differentiation of mESCs	Bradley et al., 2012
		STAT3 translocation to nucleus and NANOG negative regulation in mESC	Setati et al., 2010
		Endoderm differentiation of iPSCs	Jing et al., 2017
	HOP	STAT3 expression and phosphorylation and NANOG expression in mESCs	Longshaw et al., 2009
		Murine embryonic survival	Beraldo et al., 2013
	HSP70	Surface marker of pluripotency in hESCs	Alekseenko et al., 2012
		Differentiation of mESCs	Baharvand et al., 2008; Battersby et al., 2007; Saretzki et al., 2007
		Differentiation and survival of iPSCs	Brodarac et al., 2015
		Early differentiation of hESCs and mESCs	Park et al., 2011
	HSP60	OCT4 expression, proliferation, self-renewal and survival of mESCs	Seo et al., 2018
	HSP40	mESCs differentiation into smooth muscle cells Endoderm differentiation marker	Wong et al., 2014; Wang and Gudas, 1988
	HSP27	NANOG inactivation and neuronal differentiation of human placenta-derived cells	Cheng et al., 2016
Proteasome related	PSMD14	OCT4 regulation in mESCs and iPSCs	Buckley et al., 2012
	FBXW7	Negative regulation of c-MYC protein stability in mESCs and iPSCs	
	NRF2	Pluripotency maintenance in hESCs and iPSCs	Jang et al., 2014
	PSMD11	Functional proteasome complexes formation in hESC and iPSC	Vilchez et al., 2012
	F0X04	PSMD11 expression regulator in hESCs and iPSC	
	L1TD1	Downregulation leads to decrease in SOX2 and PSMD11 of hESC	Emani et al., 2015
	C0PS2	NANOG protein stability regulator of mESCs	Zhang et al., 2016
	WWP2	Promotes OCT4 and SOX2 proteasome degradation in mESCs	Xu et al., 2004, 2009, Fang et al., 2014
	SET7	SOX2 methylation and proteasome degradation promotion in mESCs Transcriptional activity inhibition in mESCs	Fang et al., 2014
	AKT1	SOX2 phosphorylation and proteasome degradation prevention in mESCs	
	UBR5	Proteostasis machinery regulator in hESCs and iPSCs	Koyuncu et al., 2018
	FBXW8	Polyubiquitynates NANOG and mESCs	Kim et al., 2014
	USP21	NANOG protein stabilization in mESCs and hESCs	Jin et al., 2016; Liu et al., 2017
	USP26	NANOG and SOX2 genes inhibition in hESCs	Ning et al., 2017

(Zhang et al., 2016). Further, the levels of NANOG remain unaltered after the replacement of four lysine residues for arginine in its C-terminal domain and subsequent KD of COPS2, indicating this region is a strong candidate for ubiquitination, serving as a signalization for UPS degradation (Zhang et al., 2016).

More specifically, it has been previously demonstrated that OCT4 can be post-translationally modified by ubiquitin in both mESCs and hESCs. WWP2 protein was identified as the first to post-translationally modify OCT4, functioning as an E3 ligase (Xu et al., 2004). WWP2 promotes degradation of OCT4 in a dosage-dependent and also enzymatic activity-dependent manner through the 26S proteasome (**Figure 1A**), since OCT4 protein level progressively decreases with the increase of WWP2 expression level, and WWP2 silencing (by iRNA and shRNA) elevates OCT4 levels (Xu et al., 2009). Further, OCT4 has been shown to suffer SUMOylation at lysine residue 118 (SUMO-1 acceptor site) in mESCs, and the disruption of this modification can lead to the degradation of OCT4 by 26 proteasome and consequent impairment in self-renewal (Zhang et al., 2007).

Interestingly, evidence shows that WWP2 also plays a role in the regulation of SOX2. WWP2 HECT domain recognizes methylation on lysine 119 (K119me) of SOX2, modification that stimulates SOX2 ubiquitination and subsequent degradation via proteasome pathway (Fang et al., 2014). Treatment with MG132, a potent proteasome inhibitor, led to prominently increased levels of SOX2 K119me. The methylation in SOX2 is catalyzed by the enzyme SET7 which, intriguingly, also inhibits the transcriptional activity of SOX2 (Fang et al., 2014). On the other hand, in ESCs, direct phosphorylation of SOX2 at threonine 118 by AKT1 is a prevalent protection mechanism acting in SOX2 protein stability (Jeong et al., 2010), and inhibits methylation by SET7 at K119, stabilizing SOX2 levels, being crucial in aspects such as self-renewal and differentiation potential (Fang et al., 2014). The mechanism of AKT-mediated phosphorylation as a protector for degradation by the UPS has also been recently identified in esophageal cancer stem cells, where phosphorylation of SOX2 on threonine 116 protects the degradation mediated by the ubiquitin E3 ligase UBR5 (Wang et al., 2019). It is interesting to note that UBR5 has also been identified as an important factor

in the regulation of proteostasis in hESCs and iPSCs. UBR5 is upregulated in hESCs and iPSCs derived from Huntington disease patients (Koyuncu et al., 2018). Both protein and mRNA levels are downregulated as these cells undergo differentiation (Koyuncu et al., 2018), indicating an important role of this protein in PSCs in health as well as disease. Given that PSCs and cancer stem cells share several features, the importance of UBR5 and other E3 ligases regulating the levels of TFs in PSCs should be further studied.

NANOG was previously described as a target for polyubiquitylation by the F-box protein family member FBXW8, and consequent degradation by proteasome (Kim et al., 2014). When phosphorylated by ERK1, NANOG binds to FBXW8, and experiments using mESCs and hESCs overexpressing FBXW8 resulted in a decrease in the half-life of endogenous NANOG. On the other hand, evaluation of alkaline phosphatase expression, a known pluripotency indicator, in FBXW8 KD cells, indicated decreased differentiation (Kim et al., 2014). The results suggest a key role of FBXW8, as a E3 ligase, in the regulation of NANOG levels in PSCs, by controlling its degradation.

The role of deubiquitinases (DUBs) started to be investigated in the context of proteostasis maintenance in PSCs. In brief, DUBs remove ubiquitin from protein substrates in order to maintain their targets stability. A recent study in mESCs, scanning for potential deubiquitinases regulators of NANOG, found that deubiquitinase ubiquitin-specific protease 21 (USP21) stabilizes NANOG protein levels removing K48-linked polyubiquitylation (Liu et al., 2017). USP21 directly interacts with NANOG through its ubiquitin carboxyl-terminal hydrolase domain (UCH), and the phosphorylation of USP21 by ERK1 prevents this binding, leaving this site exposed for ubiquitination, ultimately leading to NANOG degradation by the UPS (Jin et al., 2016). Another USP protein was recently reported as an indirect regulator for pluripotency TFs expression. USP26 inhibits expression of pluripotency core genes by physically binding to the members of the Protein Regulator of cytokinesis 1 (PRC1) complex, CBX4, and CBX6, preventing K48-linked polyubiquitination in these targets (Ning et al., 2017). The accumulation of CBX4 and CBX6 inhibits the expression of the SOX2 and NANOG genes, increasing the occupancy of their promoters, leading to reduction in pluripotency (Ning et al., 2017).

A summary of the proteasome-related proteins presented in this section and their function in PSCs can be found in **Table 2**. The evidence presented here points out the great relevance of studying the UPS in the context of PSCs, both as a possible model to better understand this system in health and diseases, and as a means of understanding the mechanisms governing the unique biology of these cells. Although many studies have been, and are still being conducted to explore these aspects, much remains to be described. It will be very interesting to follow what will be done in order to better understand the direct or indirect relationship between the great number of molecules involved in the UPS with the regulation of pluripotency TFs levels, or with other mechanisms important for PSCs maintenance, such as cell cycle control or self-renewal.

HISTONE CHAPERONES IN PSCs

As mentioned before, several factors affect the expression of pluripotency genes, and epigenetic modulations of transcription have been broadly studied to gain insight into the mechanisms which rule the rapid proliferation and turnover of pluripotent cells into differentiation. In the context of chaperone proteins, histone chaperones have recently been identified as important factors in regulating pluripotency. Here, we discuss the importance of histone chaperones in the modulation of transcription in PSCs, mainly ESCs, and their role in maintaining pluripotency through epigenetic modifications.

Chromatin in eukaryotes is organized in complexes called nucleosomes composed of 147 pairs of bases of DNA associated with a core of small basic proteins, the histones, which form an octamer of two copies of each protein H2A, H2B, H3, and H4, binding a linker histone H1 (Biterge and Schneider, 2014; Tessarz and Kouzarides, 2014). Histones can be canonical, essentially expressed during S-phase and incorporated to the nucleosome during DNA replication, or replacement histones (known as histone variants) which are incorporated into chromatin by specific histone chaperones during the cell cycle and can interact with several chromatin modifiers modulating the chromatin conformation (Biterge and Schneider, 2014). Beyond architectural functions, variant histones can regulate transcription, DNA repair and replication through covalently modifications at their flexible N- or C-terminal tails and globular domains, modulated by chromatin-modifying enzymes, which lead to a more open conformation of chromatin and allow DNA interaction with several molecules, including TFs (Strahl and Allis, 2000; Choi and Howe, 2009).

Histone chaperones are molecules that associate with histones and present an important role in histones dynamics. These chaperones are responsible for the transferring of histones to the DNA, and they can modulate histones modifications as acetylation and methylation or remodeling nucleosomes during transcription, among other important functions. Histone chaperones can work single, as the chaperone anti-silencing function 1 (ASF1), or form complexes as chromatin assembly factor 1 (CAF1) and the facilitates chromatin transcription (FACT), presenting relevant roles in post-translational histone modifications (De Koning et al., 2007; Tessarz and Kouzarides, 2014).

Embryonic stem cells present rapid changes in transcription associated with transition from pluripotency to a more differentiated state, therefore their chromatin is characterized by an open state with a less condensed structure and predominance of variant histone modified post-translationally (i.e., methylation and acetylation of H3K4) involved in transcription activation (Gaspar-Maia et al., 2011). One of the most important mechanisms by which ESCs maintain the open chromatin state is through the deposition of specific histone variants, for example H3.3, which is located at the −1 position in promoters of genes expressed in ESCs, and is commonly associated with more active transcription and decreased methylation of H3K9, a mark of condensed chromatin (heterochromatin) (Goldberg et al., 2010; Schlesinger et al., 2017). The chaperone

histone regulator A (HIRA) is responsible for H3.3 deposition in pluripotent cells during replication and co-localizes with the transcriptional active form of histone H3K4 methylated (Goldberg et al., 2010), playing important roles in pluripotency and differentiation (**Figure 1A**). Moreover, HIRA is highly expressed in the promoters of developmentally regulated genes in ESCs and is necessary for H3.3 enrichment at genome-wide transcriptionally active and repressed genes in mESCs (**Figure 1A**) and neural precursor cells (Goldberg et al., 2010).

HIRA is also required in mESC for the formation of H3.3 complex with polycomb repressive complex 2 (PRC2), which control gene transcription during lineage commitment in these cells through trimethylation of lysine 27 on histone H3 (H3K27me3) (Banaszynski et al., 2013). This complex is responsible for the proper establishment of H3K27me3 at the promoters of developmentally regulated genes and in bivalent domains, characterized by the presence of the variant histones H3K4me3 (activation-associated) and H3K27me3 (repression-associated) (Bernstein et al., 2006; Banaszynski et al., 2013). HIRA can also modulate hESCs self-renewal through its interaction with prohibitin (PHB) promoting transcription of isocitrate dehydrogenase genes (IDHs), leading to the production of α-ketoglutarate, which in turn participates in metabolic processes that support pluripotency of hESCs (Zhu et al., 2017).

Runt-related transcription factor 1 (RUNX1) is essential for hematopoietic cells transition and HIRA can modulate RUNX1 targets participating of mESCs differentiation process to hemogenic endothelium through its interaction with RUNX1 and deposition of H3.3 variant (Scambler et al., 2015). Additionally, H3.3 deposition by HIRA during early embryogenesis is required for developmental reprogramming, since the loss of HIRA in female mice impairs the deposition of paternal core histone and compromise the reactivation of maternal genome (Lin et al., 2014).

Transcriptional regulator ATRX (ATRX) is a member of SNF2 family of chromatin remodeling factors and presents histone chaperone activity, forming a chromatin-remodeling complex with the death domain-associated protein DAXX. ATRX and DAXX associate with H3.3 in a HIRA-independent manner modulating H3.3 deposition at telomeres and repression of telomeric RNA in mESCs (Goldberg et al., 2010). DAXX functions as an H3.3-specific chaperone in mESCs assembling H3.3/H4 tetramers on DNA templates, and the ATRX–DAXX complex modulates the remodeling of H3.3-containing nucleosomes bounding to telomeric chromatin or pericentric repeats (Lewis et al., 2010). Interestingly, it was demonstrated that DAXX and ATRX complex are responsible for H3.3 incorporation in transposable elements containing long terminal repeats, which present regions enriched with both H3.3 and H3K9me3, those able to regulate adjacent and endogenous genes in mESCs (Elsässer et al., 2015).

Anti-silencing function 1 is the most conserved chaperone of histone 3 and 4 and is associated with nucleosome assembly, transcriptional downregulation and DNA damage response (Thuret et al., 2005). In murine, two paralogous genes *ASF1a* and *ASF1b* are present; ASF1a depletion is embryonic lethal while ASF1b was correlated with differentiation potential during early embryonic stages and gonad development (Messiaen et al., 2016). Additionally, it has been demonstrated that ASF1 binds histones H3.1 and H3.3 in mESCs (Lewis et al., 2010). In hESCs, ASF1a can affect the expression of pluripotency genes through the acetylation of H3K56, a histone state that represents the epigenetic mark of hESC (**Figure 1B**; Gonzalez-Muñoz et al., 2014). Interestingly, when the TFs NANOG, SOX2 and OCT4 bind to their target gene promoters it is common to observe the presence of the histone variant H3K56ac at these locations and, since ASF1 is required for the acetylation of H3K56, ASF1 depletion leads to H3K56ac decrease, and, consequently reduces expression of pluripotency markers and promotes differentiation (Tan et al., 2013). Moreover, it was demonstrated that the induced expression of both ASF1a and OCT4 in human dermal fibroblasts is able to reprogram these cells into undifferentiated iPSCs (**Figure 2**; Gonzalez-Muñoz et al., 2014).

Chromatin assembly factor-1 (CAF-1) complex is formed by 3 subunits P150, P60, and P48/RbAp48a and is related to processes involved in DNA synthesis and repair (De Koning et al., 2007), and also chromatin remodeling in ESCs (Houlard et al., 2006). CAF-1 is the histone chaperone that mediates H3 deposition during S-phase, associates with H3.1, transports H3.3 in the absence of DAXX in mESC and its dominant negative leads to developmental arrest before 16-cell stage (Lewis et al., 2010; Filipescu et al., 2013). The lack of one of CAF-1 subunits (P150) induces the complete loss of H3.1 and H3.2 and impairs mouse blastocysts stage development, which present modified cellular structures such as nuclear elongation and the absence of heterochromatin foci (Akiyama et al., 2011). Houlard and Filipescu, in their respective studies, demonstrated that CAF-1 associated with histone H3.2 is responsible for heterochromatin proper architecture in early development, it may contribute to gene expression during this period of development, and is also required for early gastrulation (Houlard et al., 2006; Filipescu et al., 2013). The depletion of CAF-1 subunit P150 in mESCs affects the structure of heterochromatin, which is not observed in somatic cells, leading to its decondensation and the loss of clustering and mislocalization of pericentric heterochromatin domains (Houlard et al., 2006). Moreover, CAF-1 knockdown increases H2AX phosphorylation and the developmental arrest of mouse embryos, avoiding retrotransposons appropriate silencing and damaging genome integrity, since CAF-1 is responsible for modulating the replacement of H3.3 for H3.1/3.2 and leads to the deposition of other repressive histones as H3K9me3, H3K9me2, H3K27me3, and H4K20me3 in preimplantation mouse embryos (Hatanaka et al., 2015).

Cheloufi and colleagues recently demonstrated the participation of CAF-1 in the reprogramming of mouse fibroblasts into iPSCs, since the negative modulation of CAF-1 expression produces a decrease in somatic cells heterochromatin domains, increasing the binding of SOX2 to pluripotency specific targets and somatic cellular plasticity (Cheloufi et al., 2015; Cheloufi and Hochedlinger, 2017). Interestingly, CAF-1 suppression also allows ESCs to acquire characteristics of an early developmental

state, resembling totipotent 2-cell (2C) blastomeric stage of the preimplantation embryo (Yankulov, 2015).

The histone chaperone FACT is a complex composed by two subunits, SSRP1 and SPT16, and participates in transcription elongation (Mylonas and Tessarz, 2018). In mESC, FACT depletion causes a mis-activation of transcription start sites, which deregulates developmental and replication-associated genes leading to an increase in proliferation concomitant with neural commitment (Mylonas and Tessarz, 2018). FACT has been associated with OCT4 and its depletion damages mESC pluripotency and survival during early blastocyst stage (Cao et al., 2003; Gaspar-Maia et al., 2009; Pardo et al., 2010; Ding et al., 2012). Shakya et al. (2015) demonstrated that OCT4 recruits FACT complex, binds specifically SPT16 subunit of FACT in transcription sites to promote the removal of H3 histone from critical pluripotency targets such as OCT4, SOX2, and NANOG during reprograming. Moreover, it was demonstrated that the depletion of SPT16 subunit of FACT in mouse fibroblasts impair cellular reprograming and iPSCs generation (Shen et al., 2018).

Several other histone chaperones have presented important functions on ESCs biology and somatic cells reprograming. The mammalian high-mobility group AT-hook 2 (HMGA2) are highly expressed in ESCs and not translated in somatic normal cells, working as a replication fork chaperone stabilizing its integrity, in a DNA repair mechanism. During the proliferation of highly replicating cells as ESCs or cancer cells, HMGA2 expression is irrelevant, however, when forks are arrested; HMGA2 binds with high affinity to branched DNA, stabilizing stalled forks and preventing DNA mutations (Yu et al., 2014).

Another histone chaperone, nucleoplasmin-2 (NPM2), was recently associated with human dermal fibroblasts reprogramming. NPM2 associated with its histone variants TH2A and TH2 can improve the reprogramming modulated by OCT4, SOX2, KLF4, and c-MYC, generating iPSCs in a more naïve state compared to the classical TFs alone (**Figure 2**; Shinagawa et al., 2014; Fernández-Rivero et al., 2016). NPM2 phosphorylated form can also improve murine reprograming inducing an open chromatin structure and leading to reprogramming using only KLF4 and OCT4 (Shinagawa et al., 2014). Another NPM, the histone chaperone nucleoplasmin 3 (NPM3) is highly expressed in murine PSCs compared to differentiated cells and works as a chromatin remodeling protein, which modulates mESCs replication capacity, increasing proliferation (Motoi et al., 2008).

SPT6 is a histone chaperone and works as a transcription elongation factor in mESCs, modulating negatively H3K27 acetylation and methylation through its interaction with PRC2 core subunits, which maintains pluripotency and avoids differentiation mediated by the accumulation of H3K27me3 deposition at ESC critical super-enhancers (Robert, 2017). SET is a histone chaperone, which expression is modulated by OCT4, and is expressed during human early development and presents two isoforms, SETα and SETβ, each one controlling ESCs proliferation and differentiation, respectively (Edupuganti et al., 2017).

Finally, it is well known that PSCs present rapid turnovers to maintain an undifferentiated state or for entry into cellular commitment. Epigenetic mechanisms of gene activation that depends on histone variants allow these rapid turnovers observed in stem cells, especially PSCs, and histone chaperones are determinant for the refined work of histones modifications. Briefly, here we described how histones chaperones present relevant roles on development, pluripotency maintenance and somatic cell reprograming, as their main function is to modulate these essential epigenetic changes related to histones modifications and deposition, which have great participation on the activation and repression of TFs. **Table 1** compile the main histone chaperones and their role in pluripotency and differentiation. In addition to the histone chaperones, other chaperones are closely involved to maintain the pluripotent state, and their functions in PSCs biology will be explored in the next sessions.

PN AND HEAT SHOCK CHAPERONES IN PSCs

Elements of the PNs are enhanced in PSCs, such as chaperones and co-chaperones. Chaperones are classified in heat-shock proteins (HSPs) such as small heat shock proteins (sHSPs), HSP60, HSP70, HSP90, and HSP100. HSP70 and HSP90 are the main families of chaperones induced in response to cellular stress and they act along TFs known as heat shock factors (HSF), which regulate several genes encoding chaperones (Hartl et al., 2011; Saibil, 2013).

The heat shock factor 1 (HSF1) creates a dormant complex with HSP90 and HSP70 under balanced conditions, however, in the presence of misfolded proteins and cytotoxic protein aggregation, chaperones detach from HSF1, which then induces members of the PN to decrease production of new clients (Saibil, 2013). When the proteotoxic stress is reduced, the HSF1 complex reassembles and the system returns to balance. Likewise, other stress-inducible chaperones are expressed in the cytosol and within organelles to create arrangements that keep non-native proteins in solution and control *de novo* folding to establish functional structures (Saibil, 2013). HSPs are abundantly expressed in PSCs than in terminally differentiated cells, providing enhanced stress response competence for PSCs which is essential for the maintenance of stemness (Saretzki, 2004).

Both protein quality control and the maintenance of the PN are indispensable for cellular biology and for the health of whole organism. Important additional pathways such as the cytosolic stress response and the unfolded protein response (UPR) are part of this complex that contributes to circumvent the accumulation of misfolded molecules (Arndt et al., 2010). However, the PN is prone to failure, despite its regulatory mechanisms, and it opens a gap for pathological protein aggregate deposits. It is considered that aggregate formation confers on the disease protein a toxic gain of function (Dimant et al., 2012). Deficiencies in proteostasis related to aggregation have been shown to facilitate the development of several illnesses,

including neurodegeneration, diabetes, cystic fibrosis, and cancer (Kakkar et al., 2014). The importance of specific families of chaperones and co-chaperones in the context of pluripotency will be addressed in subsequent sessions.

HSP90-HOP-HSP70 Complex

Heat shock protein 90 (HSP90) is a highly conserved stress response protein in eukaryotic cells, being the most abundant protein in unstressed cells, modulates the activity, turnover and trafficking of several proteins and participates of signal transduction. HSP90 forms heterocomplexes with different proteins through the formation of a complex composed by HSP90 and another four chaperones – HSP70, HSP Organizing Protein (HOP), HSP40, and p23 – binding to several clients as steroid receptors. The assembly requires HSP90 and HSP70 bound by HOP and incorporates HSP40 and p23 during the process, assisting the folding and refolding of naïve proteins for properly active conformation (Pratt and Toft, 2003). This chaperone machinery is ATP-dependent, in which HSP90 binds to ATP and p23 stabilizes the complex. Both HSP70 and HSP40 also interact after ATP binding and HOP brings together both complexes preparing the chaperone machinery to receive a substrate, that can be a receptor, a protein kinase or a TF, among other proteins associated with signaling transduction (Pratt and Toft, 2003). The HSF are TFs responsible for increasing heat shock proteins expression during stress conditions and, on the other hand, HSP90 complex is responsible for sequester HSFs in normal conditions avoiding stress response activation in a feedback loop (Nichols et al., 1998).

Several studies support the importance of HSP90-HOP-HSP70 complex on PSCs biology. Recent data from our group determined that HOP, HSP70, and HSP90 are found as cargo of extracellular vesicles (implicated in intercellular communication) of mESCs, suggesting their participation in processes related to development, since the microenvironment is important for pluripotency maintenance through the modulation of several signaling pathways (Cruz et al., 2018a,b).

Constitutive HSP90 is required for mouse embryos development and maintenance of pluripotency via HOP-STAT3 interaction (**Figure 1A**); moreover, ESCs express a specific conjunct of types of chaperones related to this complex as HSP70 protein 4 (HSPA4) and HSP90β (HSPCB). In addition, stem cells including ESCs present an increase in the expression of HSP70 protein 5 (HSPA5), HSP70 protein 8 (HSPA8), and HOP (Fan, 2012). HSP70 protein 8 (HSPA8) is highly expressed on the surface of hESCs (**Figure 1B**) and co-expressed with specific stem cells markers, for example stage-specific embryonic antigen 3 and 4 (SSEA3/4) (Son et al., 2005).

HSP90 has been described in literature as an essential molecule for pluripotency maintenance. The inhibition of HSP90 expression decreases OCT4, NANOG and pSTAT3 levels and leads to mESCs differentiation, preferentially to the mesoderm layer. HSP90 associates with OCT4 and NANOG to protect them from ubiquitin proteasome degradation (**Figure 1A**) and is also able to modulate OCT4 mRNA levels supporting pluripotency maintenance (Bradley et al., 2012). During heat shock in hESCs, there is a hyperactivation of the MAP kinases and hESCs starts to differentiate through the increase of the HSF1 transcription factor, HSP90 releases HSF1 to answer the stress stimuli and HSF1 in turn binds to OCT4 promoter region leading to its repression (Byun et al., 2013).

Setati et al. (2010) described that HSP90 is responsible for STAT3 translocation to the nucleus during the activation of the JAK-STAT3 pathway, which occurs through LIF stimuli during mESCs self-renewal (**Figure 1A**). STAT3 translocation to the nucleus by HSP90 leads to STAT3 association with the NANOG promoter region modulating pluripotency of mESCs (Okumura et al., 2011). Negative modulation of NANOG transcription occurs through constitutive HSP90 sequester by TRIM8, which impairs its association with STAT3 and, consequently translocation to the nucleus, avoiding pluripotency signaling via LIF pathway (Setati et al., 2010). One of HSP90 clients is the transcription factor HNF4A, their binding in iPSCs is able to modulate specifically differentiation from these to endoderm-derived hepatic progenitor cells (Jing et al., 2017).

HSP90 partner HOP is essential for mouse development since its knockout (KO) is embryonic lethal and HOP KO mouse do not reach E10.5 development stage (Beraldo et al., 2013). HOP was suggested to modulate STAT3 phosphorylation and, through its binding to HSP90, participate in STAT3 translocation to the nucleus, being another important chaperone in mESC pluripotency by LIF/JAK/STAT3 signaling pathway (**Figure 1A**; Longshaw et al., 2009). Longshaw et al. (2009) demonstrated STAT3 accumulation into the cytoplasm, decreased phosphorylation and STAT3 mRNA levels after HOP depletion in mESC. Moreover, HOP knockdown mESCs presented a decrease in NANOG mRNA levels and decreased pluripotency observed in the formation of embryoid bodies (EBs).

HSP70 is constitutively expressed and its expression can be inducted during stress. In hESCs, constitutive HSP70 expression is slightly increased compared to somatic cells and is located into the cytoplasm and nucleus independent of differentiation status. However, in hESCs HSP70 can also be found on cell surface (**Figure 1B**), which is not observed in somatic cells, suggesting HSP70 is a possible surface marker for pluripotent cells (Alekseenko et al., 2012).

The HSPS from the family HSP70 HSPA1A (HSP70A1), HSPA1B (HSP70B1), and HSPA9A (HSP70A9, mortalin) are highly expressed in mESCs compared to differentiated cells and are associated with increased resistance of these cells against genotoxic stress (Saretzki, 2004). Additionally, it was demonstrated that during differentiation of some mESC lines there is a decrease in expression of both HSP70 and its partner HOP (Baharvand et al., 2008).

Recently, it was demonstrated that upregulation of HSP70 in iPSCs caused by stress as hypoxia promotes survival through the inhibition of apoptosis pathways, however, it also inhibits STAT3 phosphorylation leading to differentiation (**Figure 2**) and increased resistance to stress, since iPSCs are highly sensitive to conditions adverse to homeostasis (Brodarac et al., 2015). HSP70 participates in the early differentiation modulated by epigenetic factor histone deacetylase (HDAC) of mESCs and hESCs, through the activation of JNK and PI3K/AKT pathways (Park et al.,

2011). In mESC, HSP70 expression is enriched by the MAPK signaling pathway though JNK, ERK, and p38 modulating several responses to stress related to cell survival and anti-apoptosis processes (Nishitai and Matsuoka, 2008).

Differentiation of mESCs into neurogenic EBs (NEBs) leads to a decrease in the expression of mortalin, a chaperone from the family of HSP70 (HSP70A9) (Battersby et al., 2007). Another protein from HSP70 family, HSPA1b, also presents a significant decrease in its expression during differentiation and its remarkable that this downregulation occurs and is detectable before the expression of differentiation markers, supporting a role for HSP70 proteins in modulating differentiation (Saretzki et al., 2007).

HSP60, HSP40, and Small HSPs Families

HSP60, also known as HSPD1, is a mitochondrial chaperonin, involved in the synthesis and transportation of mitochondrial proteins from the cytoplasm to the mitochondria (Cappello et al., 2008). HSP60 is able to interact with different HSPs, such as HSP10, forming a complex that mediates protein folding (Okamoto et al., 2017), and with mitochondrial HSP70 (HSP70A), also known as mortalin, that have a role in cell proliferation and stress (Wadhwa et al., 2006). Studies shows that HSP60 have an important role in the biology of pluripotent cells. HSP60 deficiency in progenitor cells from the intestinal crypt compartment induces mitochondrial dysfunction, which leads to a decrease in stemness and cell proliferation (Berger et al., 2016). In mESCs, HSP60 expression decreases with cell differentiation, and its depletion caused a decrease in OCT4 expression, inhibiting proliferation and self-renewal, and increasing apoptosis in a caspase-3 independent-manner (Seo et al., 2018). Besides that, HSP60 knockdown also decreased EBs size and increased S-phase cells in mESCs (Seo et al., 2018). As seen in mouse cells, rabbit ESCs (rESCs) HSP60 levels are also increased when compared to differentiated cells, suggesting that proteins from the HSP family might have an important role in maintaining the undifferentiated status of embryonic cells (Intawicha et al., 2013). Interestingly, studies in cancer showed that *HSP60* is a target gene of c-MYC (Tsai et al., 2008), but is also able to induce c-MYC expression, as *HSP60* overexpression increased the proteins levels of c-MYC (Yan F.Q. et al., 2015).

HSP40 can interact with HSP70 and modulate its ATPase activity through stabilizing its interaction with subtracts (Prinsloo et al., 2009). HSP47, also known as SERPINH1, is required for the proper assembly of triple-helical procollagen molecules, and is highly expressed during mESC differentiation into smooth muscle cells (SMC) (Wong et al., 2014). Depletion of HSP47 leads to a decrease in the differentiation of mESC to mSMC and, in the same way, an overexpression of HSP47 leads to an increase in differentiation (Wong et al., 2014). HSP47 was identified as a marker for endodermal differentiation in mouse teratoma (Wang and Gudas, 1988). In bovine embryos, all HSP40 family members are upregulated during degeneration in an anti-apoptotic system, while normal blastocysts highly express HSP70 family members as HSPA5 and HSPA8 (Zhang et al., 2011). Both HSP40 and HSP47 are involved in the development of mouse limbs, along with other chaperones including from HSP70 family and small HSPs (Zhu et al., 2010; Yan F.Q. et al., 2015). A study using Glioblastoma Multiforme stem cells showed that HSP47 is capable of modulating TGF-β, inducing cell survivability, self-renewal, and increasing the number of stem cell-like cells within the tumor (Jing et al., 2017). Still, more studies elucidating the possible roles of the HSP40 family in the maintenance of stem cells are necessary.

The small HSP family is composed out of HSP of low molecular weight. Small HSPs present relevant roles in mouse development, for example HSP10, HSP22, and HSP25 that are involved in murine limb development (Zhu et al., 2010; Yan Z. et al., 2015). In adipose tissue-derived stem cells, HSP32, also known as hemeoxygenase-1, has its expression increased after the cells are left in 43°C for 1 h, which in turn increases the prevalence of live cells after a frozen-thawn cycle, reducing oxidative stress damage (Shaik et al., 2017). Besides that, a study in Chronic Myeloid Leukemia, a hematopoietic stem cell disease, shows that HSP32 is capable of increasing the survivability of those cells, through protection against apoptosis (Mayerhofer et al., 2008). Another small HSP with an interesting role in stemness is HSP27, also known as heat shock protein beta-1 (HSPB1). A study shows a downregulation of HSP27 after mESC differentiate into NEB (Prinsloo et al., 2009). Besides that, HSP27 is capable of regulating STAT3 expression, with their expression levels correlating directly in prostate cancer cells (Rocchi et al., 2005). HSP27 is also able to regulate EIF4E and eIF4G levels in the first trimester human placentae, indicating that HSP27 could alter placental protein translation (Shochet et al., 2016). HSP27 also seems to have an important role in neuronal differentiation, with its overexpression leading stem cells to arrest neuronal differentiation, and its expression decreasing as embryonic neuronal development occurred *in vivo* (Cheng et al., 2016). In placenta-derived multipotent cells (PDMCs) HSP27 inhibition leads to NANOG cleavage mediated by caspase-3 activation (Cheng et al., 2016).

In zebrafish development both HSP27 family members, HSPB7 and HSPB12, are modulated by GATA4 protein and modulate cardiac development, since their depletion leads to a disruption in normal cardiac morphogenesis perturbing Kuppfer's vesicle morphology (Rosenfeld et al., 2013). Indeed, HSPB7 is highly expressed in cardiac cells and its depletion in mouse lacks the normal development of the heart, leading to embryo lethality before embryonic day 12.5 (Wu et al., 2017). HSPB1 (HSP27 family) also presents an important role in zebrafish development modulating the growth of myofibrils of skeletal muscle (Middleton and Shelden, 2013). In avian blastoderms, the expression of the sHSP HSP25 is required during the embryo development for the expression of pluripotency genes and for resistance against apoptosis (Hwang et al., 2016).

In light of all these findings, it is remarkable that the participation of heat shock proteins in the biology of PSCs. HSPs can modulate several signaling cascades including pluripotency essential pathways. They also modulate both expression and localization of TFs and relevant proteins, and their expression is essential for the development of the embryos of many species. Together, these studies demonstrate the importance of stress response and HSPs function in development and pluripotency maintenance of PSCs.

PERSPECTIVES

A better overview of the nature of pluripotency holds great promise for therapeutic approaches. Unveiling the exact molecular mechanisms that govern the ability of ESCs and iPSCs to differentiate in all cell types to give rise to an adult organism is essential to exploit these cells both in regenerative medicine and to model human diseases pathogenesis including cancer and neurodegenerative disorders.

A regulatory core (NANOG, OCT4, and SOX2) and other TFs, epigenetic regulators and specific signaling pathways are key elements to orchestrate pluripotency. Although these well-established TFs are of undeniable importance for pluripotency maintenance, the search for additional TFs that are able to interact with core factors to regulate processes related to pluripotency should be enhanced, and the role of an expanded core has just begun to be dissected. Moving forward to the debate on the importance of TFs for pluripotency and stemness regulation, this will be a fruitful area for further research.

As presented here, emerging evidence points out the network plasticity of chaperones controlling the activities of key players involved in pluripotency maintenance. Chaperones play roles from shaping chromatin dynamic to controlling transcriptional regulation of pluripotency genes, to the assistance of proper folding of these genes when translated into proteins in different PSCs models (**Figures 1**, **2**). The broad spectrum of chaperones activities in the essential processes of stemness control reveals that stem cells exhibit intrinsic proteostasis mechanism, which can be included as a component of the pluripotency regulatory network. In somatic cells a collapse in proteostasis underlies several diseases including neurodegenerative disease and cancer, conversely, PSCs exhibit the remarkable capacity to correct and repress proteome imbalance, indicating that additional investigation is required for an in-depth understanding of enhanced protein homeostasis in stem cell biology.

This review brings together classical and recent research on the control of pluripotency, going through broad important cellular processes related to this unique and promising feature. The diversity of these processes embraces different levels of cellular regulation and shows how complex is the understanding of the pluripotency context.

AUTHOR CONTRIBUTIONS

CF and RI conceived the presented idea and proof outline, did the literature review, organized the tables, and wrote distinct topics. MP did the literature review and wrote a topic. MM-E did the literature review, wrote a topic, and prepared the illustration (art-**Figures 1**, **2**). ML conceived the presented idea and proof outline, wrote topics, and reviewed the manuscript.

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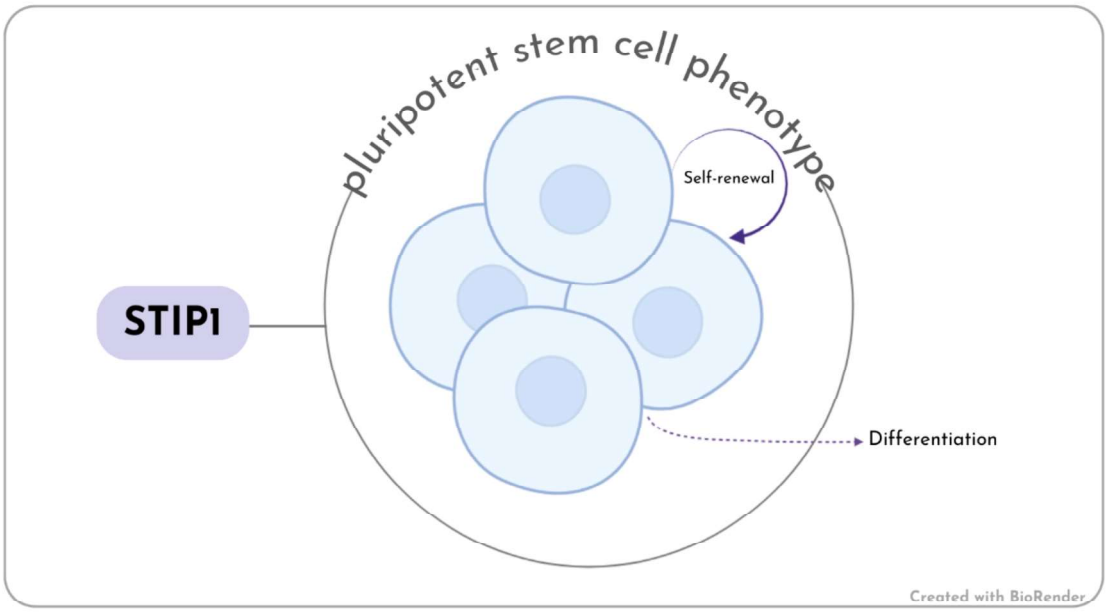
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



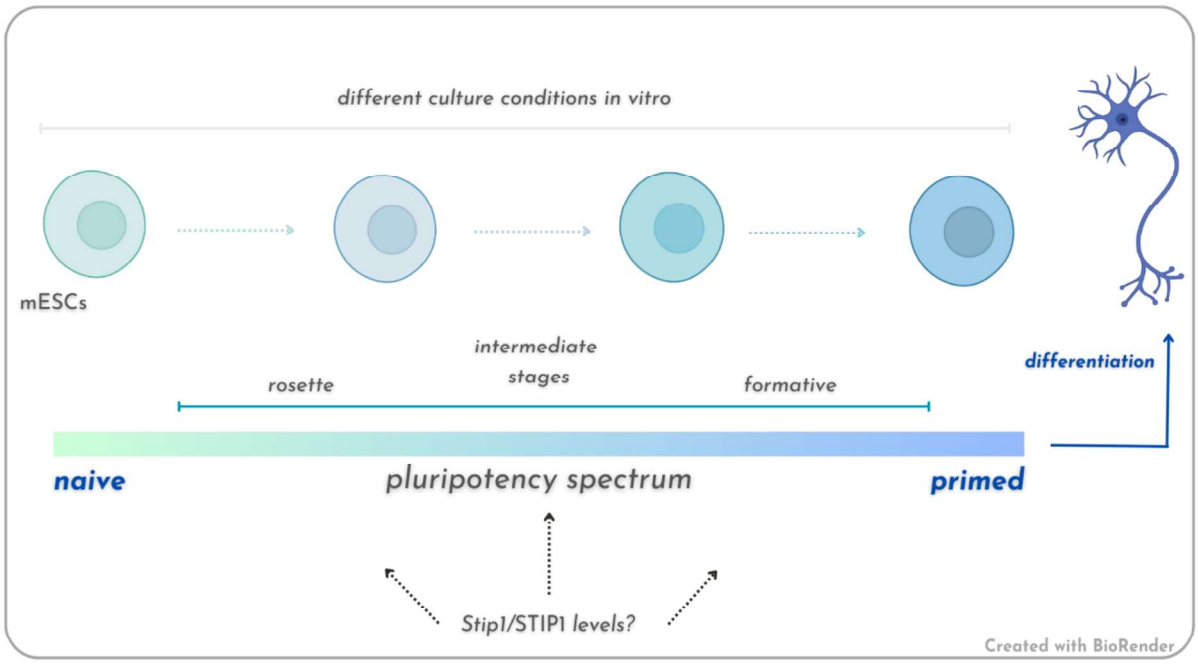
4. Chapter II

STIP1 is a stemness regulator in mESCs

Original research article entitled ***The co-chaperone stress-inducible phosphoprotein 1 is a critical stemness regulator in mouse embryonic stem cells and early mammalian development*** submitted to Stem Cells on March 5, 2024

The current chapter corresponds to the manuscript that compiles the main results obtained during my PhD. This paper was submitted to Stem Cells on **March 5th 2024**, and is currently under peer review. In addition to summarizing my main results, the article includes the work of other researchers (listed as co-authors). When I started my activities in Professor Marilene's lab, she had just returned from a research internship abroad, in the laboratory of Dr. Marco A. M Prado and Dr. Vania Prado (Robarts Research Institute - The University of Western Ontario, London, Canada). During this stay, Professor Marilene derived the mESCs lines used in this research. Additionally, she obtained confocal microscopy images, that were also included in this article and used for subsequent quantifications (performed by the group in Brazil). Professor Marilene also started the molecular characterization process of some of these cell lines (gene expression/RT-qPCR and Western Blotting experiments in mESCs wild-type and STIP1$^{\Delta TPR1}$). Of note, several of these experiments were repeated in Brazil for confirmation of these results in additional cell lines. Moreover, the article also brings together the main results of another PhD student of Professor Marilene (Supplementary Figure 3). Finally, during my PhD, I had the privilege of working with four undergraduate students, three of whom became my colleagues. Their projects were directly aligned with my research project, and their contribution during some of the analyses described here was crucial for the completion of this work.

The main objective of this study was to investigate the patterns of expression and significance of *Stip1*/STIP1 during mammalian early development, using mice as a model. Moreover, the aim was to derive and characterize mESC lines with differential STIP1 expression and study the phenotypes of these cells.



5. Chapter III

mESCs subjected to different culture conditions have altered phenotypes

Rationale

A comprehensive understanding of pluripotency entails unravelling the subtle differences in cellular states that arise during the early stages of embryonic development. Following the isolation of the first mESCs lines by Evans and Kaufman in 1981, researchers encountered an important challenge: how to effectively cultivate these cells *in vitro* while maintaining properties similar to those of the ICM of blastocysts. Initially, ESCs were grown on a layer of fibroblasts using conditions analogous to the cultivation of embryonal carcinomas (Martin & Evans, 1975; Damjanov, 2005). Despite initial success with this approach, outcomes were often biased by co-cultivation practices and the different components used in cell culture, posing difficulties in elucidating the mechanisms of pluripotency (Martello and Smith, 2014). Consequently, researchers needed to explore alternative culture conditions and assess how they could influence the PSCs phenotype.

Research advancements in the late 1980s led to the first successful ESC cultures without the need for co-cultivation with fibroblasts, leading to the isolation of molecules, such as LIF, responsible for maintaining pluripotency (Smith and Hooper, 1987; Williams *et al.*, 1988; Gearing *et al.*, 1991). The discovery of molecules like LIF has enabled the initiation and progression of studies on the molecular mechanisms underlying pluripotency. Moreover, the isolation of hESCs (Thomson *et al.*, 1998) revealed additional molecules that could be used in the maintenance of these cells. This is because, despite sharing many characteristics, mESCs and hESCs exhibit particularities in the signalling pathways that sustain their pluripotent phenotypes (Ginis *et al.*, 2004), as do other species (Skottman and Hovatta, 2006; Tancos *et al.*, 2012; Solto *et al.*, 2021; Balbasi *et al.*, 2022; Wei et al., 2023). For instance, while LIF is crucial for maintaining mESCs, it does not have the same effect on hESCs, in which the fibroblast growth factor 2 (FGF2)/activin A pathway is more relevant (Thomson *et al.*, 1998; Dahéron *et al.*, 2004; Hirai *et al.*, 2011).

LIF was the first of the isolated molecules used for *in vitro* culture of mESCs. Its signalling pathway initiates upon LIF binding to a cell surface heterodimer receptor composed of the LIF receptor (LIFR) and glycoprotein 130 (gp130). (Gearing *et al.*, 1991). This interaction causes the recruitment of Janus kinases (JAKs) and the autophosphorylation of this protein, consequently allowing this tyrosine kinase to also phosphorylate specific sites in the cytoplasmic portion of gp130 (Stahl *et al.*, 1995). Upon phosphorylation, essential signalling cascades are activated, including JAK/STAT3 (Niwa *et al.*, 1998; Matsuda *et al.*, 1999). This pathway is initiated by the phosphorylation of gp130 docking sites by JAKs, leading to the

recruitment of STAT3, which is then phosphorylated and forms a homodimer. This dimer is subsequently translocated to the nucleus, acting as a transcription factor, directly binding to DNA, and regulating the expression of specific genes. Among these are crucial molecules for pluripotency maintenance, such as *Klf4*, *Tfcp2l1*, and *Nanog* (Onishi *et al.*, 2015).

The mechanisms by which STAT3 dimers are formed, stabilized, and translocated to the nucleus are still partially unknown. Remarkably, Setati and collaborators (2010) showed that mESCs cultured in the presence of LIF presented higher rates of the formation of a complex between HSP90 and STAT3 (Setati *et al.*, 2010). It is hypothesized that STAT3, as a dimer or as a monomer, forms a complex with HSP90 and STIP1, and this complex would be transported to the nucleus (Setati *et al.*, 2010). Interestingly, a decrease in STIP1 leads to a concomitant reduction of STAT3 levels and an accumulation of this protein in the cytoplasm of mESCs, demonstrating the importance of STIP1 in STAT3 nuclear translocation (Longshaw *et al.*, 2009).

As research on the molecular signaling involved in maintaining mESCs progressed, our understanding of the factors required for their culture has also advanced. The combination of a potent MEK inhibitor (PD0352901) with a GSK3 inhibitor (CHIR99021) demonstrated efficacy in sustaining the pluripotency and self-renewal of mESCs, both in the presence and absence of LIF, across multiple passages (Ying *et al.*, 2008; Lanner and Rossant, 2010). Referred to as "2i," this dual-inhibitor formulation has emerged as a pivotal tool in culturing mESCs, often utilized alongside other inhibitors and LIF. Understanding the *in vitro* specificities of mESCs allowed the focus on investigating the plurality of pluripotent stages. These different phases of pluripotency make a parallel with the progression of embryonic stages (Nichols and Smith, 2009). The pluripotent state observed in naïve cells mirrors the cells found in the embryo's ICM (Furlan *et al.*, 2023). This state represents a foundational stage of development, as it has the potential to give rise to all tissues of the fetus and form viable chimeric individuals when injected into another blastocyst. In the naïve stage, ESCs exhibit specific features, including increased expression of a distinct set of transcription factors (Nichols and Smith, 2009).

As development progresses, stem cells can be derived from the epiblast structure (EpiSCs) between E7 and E8. These cells are classified as primed, expressing the pluripotency core (*Oct4*, *Sox2*, and *Nanog*), along with a distinct set of transcription factors such as *Sox1*, *Sox17*, *Brachyury*, and *Foxa2*, frequently considered lineage-specific (Nichols and Smith, 2009; Kunath, 2011; Furlan *et al.*, 2023). They do not express or express little of the factors associated with naïve pluripotency such as *Kfl4*, *Kfl2*, and *Tfcp2l1*. This differential expression

pattern is analogous to cells found in gastrulae (E7) or progenitor cells, presenting some degree of phenotypical commitment (Kunath, 2011; Furlan *et al.*, 2023). Interestingly, primed cells cannot generate chimeric individuals through injection into mouse blastocysts, although they are still considered pluripotent, as they can generate teratocarcinomas when injected into adult mice (Nichols and Smith, 2009). Moreover, a series of events take place between the naïve and primed phases, resulting in cells within this transitional period exhibiting characteristics that do not fully align with either stage. Numerous studies have investigated the properties of cells in this range, leading to the concept of intermediate and gradual stages to elucidate the transition, particularly highlighting the rosette (Neagu *et al.*, 2020) and formative stages (Smith, 2017; Kinoshita *et al.*, 2021).

Despite advancements in elucidating the signalling pathways involved in the pluripotency spectrum, research on PSCs still faces challenges in comprehending the molecular mechanisms underlying these statuses. Therefore, our main objectives here were to contribute to the characterization of different pluripotency statuses by accessing their manifestation in our mESCs models and investigating whether STIP1 plays a role in the pluripotency spectrum.

Materials and Methods

Available upon publication of the full version of this thesis.

Results & Discussion

Available upon publication of the full version of this thesis.

Conclusions

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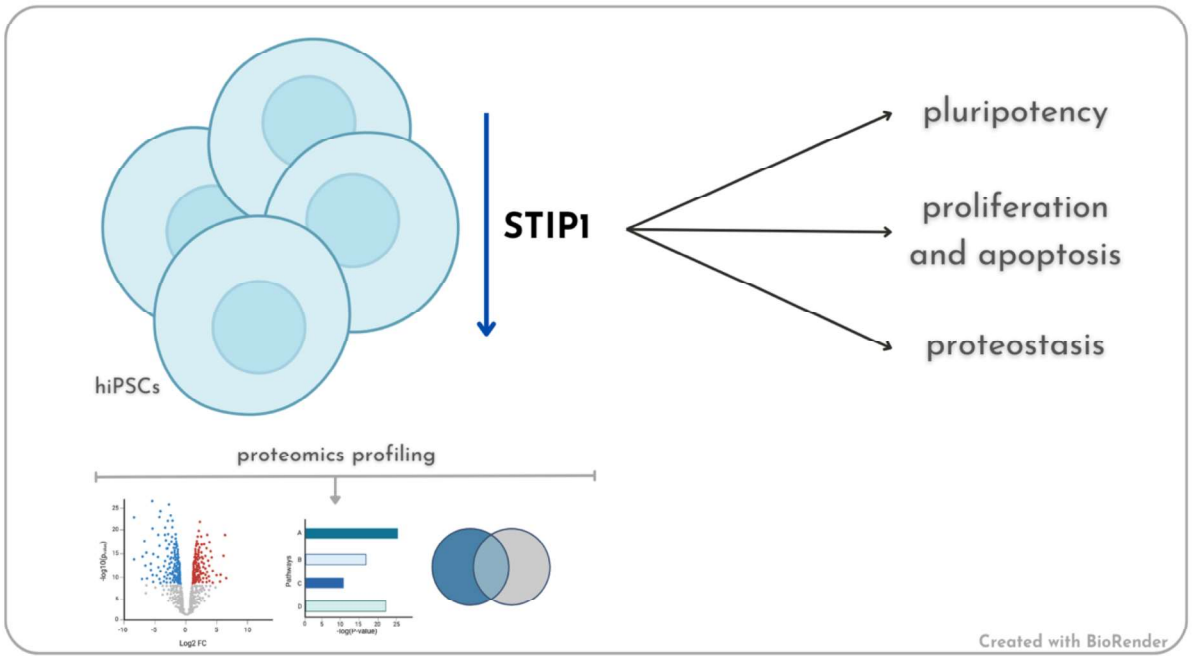

6. Chapter IV

Human pluripotent stem cells recapitulate what was observed in mESCs

Rationale

iPSCs stand out as one of the most significant biological discoveries in recent years. They serve as a valuable tool for developmental and regenerative research, fulfilling a longstanding need for an *in vitro* model to study cellular senescence and neurodegenerative diseases. Due to their intrinsic characteristics, iPSCs undergo substantial rewiring of their transcriptional and epigenetic landscape, metabolism, and proteome (Apostolou and Stadtfeld, 2020). Although, with the discovery of the Yamanaka factors, genomic control has been the primary focus of studies, post-transcriptional and post-translational features have recently been recognized as fundamental in the derivation and maintenance of iPSCs (Hao *et al.*, 2017; Li and Belmonte, 2018; Llamas *et al.*, 2020). While the protocol for derivation and reprogramming iPSCs has already been optimized, and different methods to obtain those cells were described, this process is time-consuming and has low efficiency and reproducibility (Haridhasapavalan *et al.*, 2020). Therefore, finding mechanisms to increase the productivity of iPSCs derivation also remains extremely needed. Proteostasis arose as a research field that urges more in-depth studies in the context of iPSC biology, and it could possibly impact not only the understanding of these cells but also their efficient applicability.

As ESCs, iPSCs exhibit increased proteostasis activity (Vilchez *et al.*, 2012; García-Prat *et al.*, 2017; Noormohammadi *et al.*, 2018; Fernandes et al., 2019). While cellular senescence is accompanied by a strong downregulation of proteostasis, and the dysfunction of this mechanism is a hallmark of several neurodegenerative diseases, iPSCs have more efficient mechanisms to cope with cellular senescence (Vilchez *et al.*, 2014; Hipp *et al.*, 2019). Proteostasis acts in these cells by directly regulating the levels of pluripotency factors and assisting in the folding, re-folding, proper localization, and degradation of pluripotency proteins (García-Prat *et al.*, 2017; Fernandes *et al.*, 2019; Llamas *et al.*, 2020). Additionally, iPSCs present increased proteasome activity and upregulation of several proteins involved in proteostasis, and this activity is often fundamental to the reprogramming of iPSCs (Vilchez *et al.*, 2012; Llamas *et al.*, 2020). Nevertheless, the precise mechanisms through which iPSCs prevent cellular senescence via proteostasis remain poorly understood, warranting further investigation.

So far, our results have explored the role of STIP1 in mouse early development and the regulation of several aspects of pluripotency maintenance in mESCs (**Chapters I-III**). STIP1 is an evolutionarily conserved protein, and mouse and human STIP1 are 97% similar (Honoré *et al.*, 1992). Furthermore, although STIP1 KO is lethal during the embryonic development of

mice (Beraldo *et al.*, 2013), in differentiated human cells, such as HEK293T and different types of tumor cells, the KO was not lethal (Bhattacharya *et al.*, 2020; Chao *et al.*, 2022). On the one hand, this raises the possibility that the functions of STIP1 and its essentiality in cells vary between mouse and human cells, and on the other hand indicates possible specific functions of STIP1 in undifferentiated cells. Hence, our final objective was to determine whether the observations made in mESCs would be conserved in human pluripotent cells, using hiPSCs as models. We aimed to elucidate the possible association between the levels of STIP1 expression and pluripotency maintenance in hiPSCs to better understand the regulation of proteostasis in these cells, helping to shed light on potentially neglected post-translational mechanisms and novel master post-genomic regulators of pluripotency, such as STIP1.

Materials and Methods

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Results & Discussion

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Conclusions

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7. Conclusions

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8. Perspectives

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Annexe

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Review

Extracellular vesicles throughout development: A potential roadmap for emerging glioblastoma therapies

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ABSTRACT

Extracellular vesicles (EVs) are membrane-delimited vesicular bodies carrying different molecules, classified according to their size, density, cargo, and origin. Research on this topic has been actively growing through the years, as EVs are associated with critical pathological processes such as neurodegenerative diseases and cancer. Despite that, studies exploring the physiological functions of EVs are sparse, with particular emphasis on their role in organismal development, initial cell differentiation, and morphogenesis. In this review, we explore the topic of EVs from a developmental perspective, discussing their role in the earliest cell-fate decisions and neural tissue morphogenesis. We focus on the function of EVs through development to highlight possible conserved or novel processes that can impact disease progression. Specifically, we take advantage of what was learned about their role in development so far to discuss EVs impact on glioblastoma, a particular brain tumor of stem-cell origin and poor prognosis, and how their function can be hijacked to improve current therapies.

1. Introduction

Extracellular vesicles (EVs) are membrane-delimited structures that carry several cargos, including proteins, lipids, nucleic acids, miRNAs, extracellular DNA, and even whole organelles, such as mitochondria [1,2]. EVs are believed to be released by all cell types and are highly conserved through evolution. Since their identification in the mid-1960 s, the field of research on EVs has grown exponentially, exploring their biogenesis, function in health and disease, and their therapeutical promises [2–8]. EVs have been previously considered useless cellular waste, but their current accepted role as complex intercellular means of communication highlights their importance to cell biology. This complexity translates into four ways of cargo delivery: by fusion with the cellular membrane of the target cell and transfer of the cargo; by endocytosis of the EV by the target cell and transfer of the cargo; by ligand interaction of the EV with receptors on the membrane of the target cell and subsequent signaling; or by the release of EV cargo in the extracellular space [9].

The scientific community has only begun to scratch the surface of the possibilities of different cellular interactions that EVs can exert. Despite all the work done in the last few years, specific classification of all EVs types and the terms used to define them are still a topic of discussion [10]. Therefore, the expression "EVs" corresponds to a general denomination given to several subtypes of vesicles classified according to their biogenesis, morphology, size, density, and content [4,10]. Here, we cover work regarding exosomes and microvesicles. While exosomes are generally classified as small EVs (40–160 nm) originating from the endocytic pathway, microvesicles are 50–1000 nm vesicular structures that shed directly from the plasma membrane [3,5,11]. Nonetheless, EVs correspond to a fundamental mechanism in intercellular communication.

EVs mediate cell-cell communication, transferring components from the cell of origin to target (or recipient) cells [12,13]. Their ability to transfer their heterogeneous and often selective content in physiological or pathological conditions characterize EVs as intercellular interaction vehicles [2,14]. This feature contributes to fundamental biological processes, including early development, such as maternal-fetal communication, implantation, and signaling involving stem cell fate decisions [15,16]. Moreover, EVs function is highly relevant in several diseases, including cancer [17–19].

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RESEARCH Open Access

Integrated transcriptomics uncovers an enhanced association between the prion protein gene expression and vesicle dynamics signatures in glioblastomas

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Abstract

Background Glioblastoma (GBM) is an aggressive brain tumor that exhibits resistance to current treatment, making the identification of novel therapeutic targets essential. In this context, cellular prion protein (PrP^C) stands out as a potential candidate for new therapies. Encoded by the *PRNP* gene, PrP^C can present increased expression levels in GBM, impacting cell proliferation, growth, migration, invasion and stemness. Nevertheless, the exact molecular mechanisms through which *PRNP*/PrP^C modulates key aspects of GBM biology remain elusive.

Methods To elucidate the implications of *PRNP*/PrP^C in the biology of this cancer, we analyzed publicly available RNA sequencing (RNA-seq) data of patient-derived GBMs from four independent studies. First, we ranked samples profiled by bulk RNA-seq as $PRNP^{high}$ and $PRNP^{low}$ and compared their transcriptomic landscape. Then, we analyzed $PRNP^+$ and $PRNP^-$ GBM cells profiled by single-cell RNA-seq to further understand the molecular context within which *PRNP*/PrP^C might function in this tumor. We explored an additional proteomics dataset, applying similar comparative approaches, to corroborate our findings.

Results Functional profiling revealed that vesicular dynamics signatures are strongly correlated with *PRNP*/PrP^C levels in GBM. We found a panel of 73 genes, enriched in vesicle-related pathways, whose expression levels are increased in $PRNP^{high}$/$PRNP^+$ cells across all RNA-seq datasets. Vesicle-associated genes, *ANXA1*, *RAB31*, *DSTN* and *SYPL1*, were found to be upregulated *in vitro* in an in-house collection of patient-derived GBM. Moreover, proteome analysis of patient-derived samples reinforces the findings of enhanced vesicle biogenesis, processing and trafficking in $PRNP^{high}$/$PRNP^+$ GBM cells.

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REVIEW
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Multifaceted WNT Signaling at the Crossroads Between Epithelial-Mesenchymal Transition and Autophagy in Glioblastoma

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Tumor cells can employ epithelial-mesenchymal transition (EMT) or autophagy in reaction to microenvironmental stress. Importantly, EMT and autophagy negatively regulate each other, are able to interconvert, and both have been shown to contribute to drug-resistance in glioblastoma (GBM). EMT has been considered one of the mechanisms that confer invasive properties to GBM cells. Autophagy, on the other hand, may show dual roles as either a GBM-promoter or GBM-suppressor, depending on microenvironmental cues. The Wingless (WNT) signaling pathway regulates a plethora of developmental and biological processes such as cellular proliferation, adhesion and motility. As such, GBM demonstrates deregulation of WNT signaling in favor of tumor initiation, proliferation and invasion. In EMT, WNT signaling promotes induction and stabilization of different EMT activators. WNT activity also represses autophagy, while nutrient deprivation induces β-catenin degradation *via* autophagic machinery. Due to the importance of the WNT pathway to GBM, and the role of WNT signaling in EMT and autophagy, in this review we highlight the effects of the WNT signaling in the regulation of both processes in GBM, and discuss how the crosstalk between EMT and autophagy may ultimately affect tumor biology.

Keywords: glioblastoma, autophagy, microautophagy, chaperone-mediated autophagy, epithelial-mesenchymal transition, metabolic reprograming, WNT signaling

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Review

Heat Shock Proteins in Glioblastoma Biology: Where Do We Stand?

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Abstract: Heat shock proteins (HSPs) are evolutionary conserved proteins that work as molecular chaperones and perform broad and crucial roles in proteostasis, an important process to preserve the integrity of proteins in different cell types, in health and disease. Their function in cancer is an important aspect to be considered for a better understanding of disease development and progression. Glioblastoma (GBM) is the most frequent and lethal brain cancer, with no effective therapies. In recent years, HSPs have been considered as possible targets for GBM therapy due their importance in different mechanisms that govern GBM malignance. In this review, we address current evidence on the role of several HSPs in the biology of GBMs, and how these molecules have been considered in different treatments in the context of this disease, including their activities in glioblastoma stem-like cells (GSCs), a small subpopulation able to drive GBM growth. Additionally, we highlight recent works that approach other classes of chaperones, such as histone and mitochondrial chaperones, as important molecules for GBM aggressiveness. Herein, we provide new insights into how HSPs and their partners play pivotal roles in GBM biology and may open new therapeutic avenues for GBM based on proteostasis machinery.

Keywords: heat shock proteins; chaperones; glioblastoma; therapy; stem cells; proteostasis

1. Introduction

Chaperone proteins play a fundamental role in assisting protein synthesis, folding, remodeling, targeting, and inhibition of non-functional and potentially pathogenic protein aggregate formation, being essential players for the maintenance of proteome homeostasis (i.e., proteostasis) [1]. An important and vast group of chaperones is induced by heat shock and is denominated heat shock proteins (HSPs) [2]. Besides their role in proteostasis, HSPs can also act in antigen presentation, hormone receptor assembly, secretion, and cellular trafficking [3]. On one hand, considering the many roles that HSPs are involved in, their malfunction can lead to a disruption in cell proteostasis and death [4,5]. They can also be co-opted by malignant cells to help promote their survival and progression [6]. HSPs present several well described roles in cancer origin, progression, and maintenance, able to orchestrate many different mechanisms with a large number of molecules involved, demonstrating their relevance for tumor biology and, in particular, as potential therapeutic targets.

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Unconventional Protein Secretion in Brain Tumors Biology: Enlightening the Mechanisms for Tumor Survival and Progression

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Non-canonical secretion pathways, collectively known as unconventional protein secretion (UPS), are alternative secretory mechanisms usually associated with stress-inducing conditions. UPS allows proteins that lack a signal peptide to be secreted, avoiding the conventional endoplasmic reticulum-Golgi complex secretory pathway. Molecules that generally rely on the canonical pathway to be secreted may also use the Golgi bypass, one of the unconventional routes, to reach the extracellular space. UPS studies have been increasingly growing in the literature, including its implication in the biology of several diseases. Intercellular communication between brain tumor cells and the tumor microenvironment is orchestrated by various molecules, including canonical and non-canonical secreted proteins that modulate tumor growth, proliferation, and invasion. Adult brain tumors such as gliomas, which are aggressive and fatal cancers with a dismal prognosis, could exploit UPS mechanisms to communicate with their microenvironment. Herein, we provide functional insights into the UPS machinery in the context of tumor biology, with a particular focus on the secreted proteins by alternative routes as key regulators in the maintenance of brain tumors.

Keywords: secretion, brain, cancer, ER stress, leaderless, glioma, glioblastoma

INTRODUCTION

Eukaryotic cells have developed an array of mechanisms involved in protein secretion, which plays a crucial role in cellular homeostasis and cell-to-cell communication (Sicari et al., 2019). Proteins destined for secretion to the extracellular environment are initially synthesized on ribosomes in the cytoplasm and then transported to the endoplasmic reticulum (ER) (Cavalli and Cenci, 2020) in the presence of signal peptide sequences, which have the utmost importance to direct the newly produced proteins to the ER (Rehm et al., 2001). At the beginning of protein synthesis, the 7S RNA from the signal recognition particle binds to the extremity of the polypeptide chain, which pauses the

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Review

A New Take on Prion Protein Dynamics in Cellular Trafficking

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Abstract: The mobility of cellular prion protein (PrP^C) in specific cell membrane domains and among distinct cell compartments dictates its molecular interactions and directs its cell function. PrP^C works in concert with several partners to organize signaling platforms implicated in various cellular processes. The scaffold property of PrP^C is able to gather a molecular repertoire to create heterogeneous membrane domains that favor endocytic events. Dynamic trafficking of PrP^C through multiple pathways, in a well-orchestrated mechanism of intra and extracellular vesicular transport, defines its functional plasticity, and also assists the conversion and spreading of its infectious isoform associated with neurodegenerative diseases. In this review, we highlight how PrP^C traffics across intra- and extracellular compartments and the consequences of this dynamic transport in governing cell functions and contributing to prion disease pathogenesis.

Keywords: prion; PrP^C; PrP; PrP^{Sc}; vesicles; endocytosis; exosomes; trafficking

1. Introduction

Cargo compartmentalization and transport—processes involving the formation of different types of transport vesicles within the cell for endocytic and exocytic trafficking—are essential for cell survival, signaling and homeostasis [1]. Cellular prion protein (PrP^C) is a membrane-bound glycoprotein, preferentially located in lipid raft microdomains [2], where it interacts with different partners and organizes signaling platforms, modulating many molecular mechanisms [3,4]. Nevertheless, PrP^C is also capable of shifting dynamically throughout several cellular compartments, such as the cytosol, Golgi apparatus, endoplasmic reticulum (ER), and perinuclear region [5–8]. Additionally, after protein synthesis, processing and targeting to the cell surface, PrP^C can be internalized by different types of endocytosis, being primarily found in intracellular vesicles of several stages of the endocytic process [9,10]. The type of PrP^C internalization and the organelles involved in these mechanisms may trigger numerous signaling pathways and cellular outcomes [9].

Overall, the process of endocytosis involves the formation of protein-containing vesicles from distinct plasma membrane locations and, after internalization, different sets of cargoes can be routed to recycling or degradation [1]. Endocytosis is classified into clathrin-mediated and clathrin-independent processes [1]. In clathrin-mediated endocytosis (CME), clathrin acts as a heterodimer to form a lattice-like structure that coats the forming vesicle [11]. In addition, clathrin interacts with adaptor proteins, which in turn bind to phospholipids to assist the vesicle coating and the budding process [11].

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Review

Prion Protein at the Leading Edge: Its Role in Cell Motility

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Abstract: Cell motility is a central process involved in fundamental biological phenomena during embryonic development, wound healing, immune surveillance, and cancer spreading. Cell movement is complex and dynamic and requires the coordinated activity of cytoskeletal, membrane, adhesion and extracellular proteins. Cellular prion protein (PrP^C) has been implicated in distinct aspects of cell motility, including axonal growth, transendothelial migration, epithelial–mesenchymal transition, formation of *lamellipodia*, and tumor migration and invasion. The preferential location of PrP^C on cell membrane favors its function as a pivotal molecule in cell motile phenotype, being able to serve as a scaffold protein for extracellular matrix proteins, cell surface receptors, and cytoskeletal multiprotein complexes to modulate their activities in cellular movement. Evidence points to PrP^C mediating interactions of multiple key elements of cell motility at the intra- and extracellular levels, such as integrins and matrix proteins, also regulating cell adhesion molecule stability and cell adhesion cytoskeleton dynamics. Understanding the molecular mechanisms that govern cell motility is critical for tissue homeostasis, since uncontrolled cell movement results in pathological conditions such as developmental diseases and tumor dissemination. In this review, we discuss the relevant contribution of PrP^C in several aspects of cell motility, unveiling new insights into both PrP^C function and mechanism in a multifaceted manner either in physiological or pathological contexts.

Keywords: prion protein; cell motility; adhesion; metastasis; invasiveness

1. Introduction

Cell motility is a fundamental process that entails intracellular remodeling, an intricate molecular network, and several cell–milieu interactions [1]. Motile cells are essential for several biological events, such as morphogenesis [2], angiogenesis [3], muscle contraction [4], vesicle transport [5], wound healing [6] and immune response, as well as for pathological events. Most cell types in different tissues are capable of migrating on a substrate, and cellular locomotion is governed by different processes [7]. Actin represents one of the main proteins in the cytoskeleton and its polymerization is key in the assembly and dynamic behavior of microfilaments [8]. Those microfilaments play a major role in the generation of cell polarity, since they are responsible for the formation of dynamic membrane protrusions of the cell edge, such as *lamellipodia*, *filopodia*, cell cortex, microvilli, as well as stress fibers that generate focal adhesions (FA) to the substratum, and in cytokinesis for cell division [9].

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