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Identifying the molecular players associated with transition between pluripotent and totipotent-like state

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Dedication

I would like to dedicate my PhD thesis to people who mean the world to me, but I do not tell them enough:

My mother

whose unconditional love and support is against reason...

My father

who always tried to guide me through the right direction, no matter how hard it is…

My husband and **my best friend**

For being always in the right place at the right time, catching me when I fall down and making me stand my ground. For believing in me, hearing my thoughts and knowing me inside out and finally for being my living hero…

You are my guardian angels!

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1. List of Abbreviations

2C	Two-cell
2i	2 inhibitors, GSK-3 and MEK
53BP1	53 binding protein 1
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia mutated and Rad3 related
ATRIP	ATR interacting protein
BMP	Bone morphogenic protein
CAF1	Chromatin assembly factor-1
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
Cdx2	Caudal type homeobox 2
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DDT	DNA damage tolerance
DEGs	Differentially expressed genes
DMEM	Dulbecco‟s Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Dox	Doxycycline
DSB	Double strand break
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid disodium salt
Eif1a	Eukaryotic translation initiation factor1A
EM	Electron microscope
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cells

FACS	Fluorescence activated cell sorting
Fgf	Fibroblast Growth Factor
GFP	Green Fluorescence Protein
HRR	Homologous recombination repair
Hrs	Hours
IAP	Intracisternal A particles
ICM	Inner Cell Mass
KDM1A	Lysine-specific histone demethylase 1A
Klf4	Krupple-like factor 4
KRAB	Kruppel- associated box
LIF	Leukemia inhibitory factor
LSD1	Lysine-specific histone demethylase 1
MAPK	Mitogen activated protein kinase
Mcm	Mini-chromosome maintenance
MEK	Mitogen-activated extracellular signal-regulated kinase
MuERV-L	Murine endogenous retrovirus-like
NEAA	Non-essential amino acids
NHEJ	Non-homologous end joining
NPC	Neural progenitor cells
NS	Not significant
Oct4	Octamer-binding transcription factor 4
ORC	Origin recognition complex
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-OH-kinase
Pol	Polymerase
PrE	Primitive endoderm
Pre-RC	Pre-replication complex
PSC	Pluripotent stem cells
qPCR	Quantitative polymerase chain reaction
RFC	Replication fork complex

RNA	Ribonucleic acid
RPA	Replication protein A
RS	Replication stress
SCNT	Somatic cell nuclear transfer
Sox2	SRY- box containing gene 2
ssDNA	Single stranded DNA
STAT3	Signal Transducer and Activator of Transcription 3
T-SCE	Telomere sister chromatid exchange
Tcstv	Two-cell-stage, variable group
TE	Trophectoderm
TET	Ten eleven translocation factor
TRIM	Tripartite motif
UV	Ultraviolet radiation
WT	Wild type
ZGA	Zygote genome activation
ZP	Zona pellucida
Zscan4	Zinc finger and SCAN domain containing 4

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3. Abstract

The consequence of unrepaired damage could be very serious during embryonic development as it could transmit to the large populations of cells including germ lines subsequently leading to teratogenicity and/or embryonic lethality. Therefore, it is vital for early embryonic cells to respond efficiently to genotoxic stress through coordinated and integrated DNA damage repair pathways.

The zygote and its daughter cells (2-cell-stage blastomeres) are the only known totipotent cells as they are capable of generating an entire organism while inner cell mass (ICM)-derived embryonic stem cells (ESC) are regarded as pluripotent because they have the ability to contribute to embryonic but not extra-embryonic tissues. Recent studies have identified 2C-like cells (also known as metastable state) as a rare transient cell population (~1-5%) within mouse embryonic (ESC) and induced pluripotent stem cell (iPSC) cultures that express high levels of transcripts also found in totipotent two-cell (2C) embryos. Notably, nearly all cultured PSCs cycle in and out of this metastable state, at least once during nine passages that is also accompanied by the transient transcriptional activation of 2C-spesific genes including MuERV-L and Zscan4. Interestingly, the remarkable genomic stability of mouse pluripotent stem cells has been recently linked to the transient bursts of Zscan4 (a well-known 2C marker) expression during this metastable state. Zscan4 knockdown in PSC shortens telomeres, increases karyotype abnormalities and spontaneous sister chromatid exchanges that subsequently leads to crisis by passage eight. These findings suggest that transition to 2C-like state in PSC culture is critical to maintain the genomic stability of these cells.

Although the extended developmental potential of 2C-like cells in generating embryonic plus extra-embryonic tissues as well as their higher reprogrammability through

SCNT assay has been explored by several groups, the molecular mechanism underlying the transition to 2C-like cells is not well understood. Recent reports show that cultured ESCs and mouse embryos suffer from Ataxia telangiectasia and Rad3 related (ATR) dependent constitutive replication stress which is associated with H2AX phosphorylation and chromatin loading of the ssDNA-binding proteins RPA and RAD51. Hence, given the importance of transition to 2C-like state for maintaining ESC genome stability, I hypothesized whether the transition into the metastable state is regulated through activation of DNA damage signaling pathways. Here, I found that ATR mediated DNA replication stress response triggers transition to 2C-like state in ESC. Importantly, through activation of DNA replication stress response, PSC exhibit functional and transcriptional features of 2C-like state. Overall our finding suggests a novel ATR dependent transcriptional regulation that ensures genomic integrity during a period of extreme proliferation and differentiation.

4. Introduction

4.1. Early mouse embryo development

4.1.1. Pre-implantation mouse embryo development

The first phase of embryonic development from fertilization till blastocyst formation, so-called pre-implantation stage is of immense importance for mammalian life since it is carefully orchestrated sequence of events that makes the first cell fate decisions. During pre-implantation period, embryo progressively develops more differentiated cells at the expense of more restricted developmental potency.

Upon fertilization, diploid one-cell (1C) embryo which is denoted a Zygote is produced. Few hours later, the first mitotic division of the zygote occurs which cleaves the 1C embryo to two identical daughter cells. The zygote and its daughter cells (2-cell-stage blastomeres) are the only known totipotent cells (by definition), which can generate an entire organism (Macfarlan, Gifford et al. 2012, Ishiuchi, Enriquez-Gasca et al. 2015, Zhou and Dean 2015). After two more rounds of cleavage, the 8-cell embryo is followed by compaction and polarization of the cells either toward inner or outer part of the embryo through symmetric and/or asymmetric cell divisions to form morula that by embryonic day 3.5 (E3.5) develops to the blastocyst (Chazaud and Yamanaka 2016). Blastocyst is an oval shaped structure with a fluid-filled cavity named blastocoel consisting of two distinct cell types namely inner cell mass (ICM) and Trophectoderm (TE). ICM and Trophectoderm have different transcriptional signature. While Octamer 4 (Oct4), Nanog, and SRY- box containing gene 2 (Sox2) play an essential role in the establishment of pluripotency, TE cells development depend on Caudal type homeobox 2 (Cdx2) transcription factor (Strumpf, Mao et al. 2005, Thomson, Liu et al. 2011). Subsequently, ICM cells differentiate to epiblast cells (EPI), which in turn develop all three embryonic germ layers

of the fetus, namely Endoderm, Ectoderm and Mesoderm and also primitive endoderm (PrE) cells that express Gata4 and Gata6 and contribute to formation of yolk sac (Koutsourakis, Langeveld et al. 1999). TE plays a key role in implantation of the embryo into the uterus wall and it is precursor of placenta, an essential organ ensuring fetal development (Chazaud and Yamanaka 2016). Inner cell mass (ICM)-derived embryonic stem cells (ESC) are regarded as pluripotent because they have the ability to contribute to all embryonic lineages but not to TE and extra-embryonic tissues (Macfarlan, Gifford et al. 2012, Ishiuchi, Enriquez-Gasca et al. 2015, Zhou and Dean 2015). At day 4.5 (E4.5) embryo fractures the surrounding zona pellucida (ZP) known as "hatching" and implants to the uterine to continue following phases of proper embryo development (Rossant and Tam 2009).

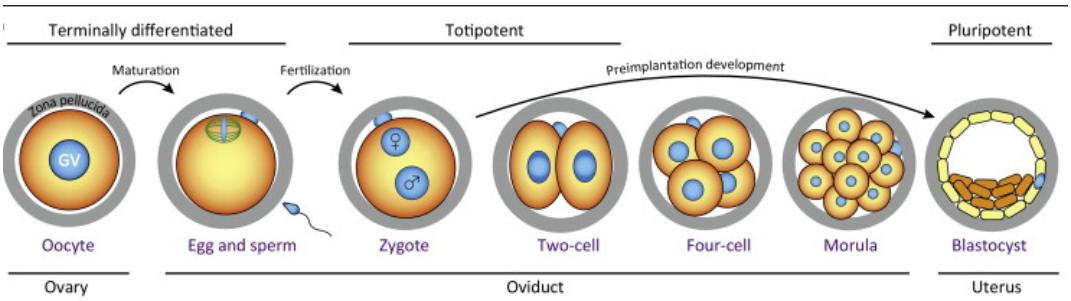


Fig 1.1. Mouse preimplantation development and maternal-to-embryonic transition.

After ovulation from the ovary into the oviduct, terminally differentiated mature eggs (surrounded by the extracellular zona pellucida) are fertilized by sperm to establish totipotent zygotes that divide during pre-implantation development to become blastocysts prior to implantation in the uterus at embryonic day 4.5. Upon fertilization, stored maternal factors activate the embryonic genome to trigger maternal-to-embryonic transition that results in formation of a totipotent zygote. Adapted from (Zhou and Dean 2015).

4.1.2. Pluripotency

4.1.2.1.ESC

Embryonic stem cells (ESC) are the first *in vitro* pluripotent cells that were derived from ICM of the blastocyst and established artificially by culturing them on inactivated mouse embryonic fibroblast (MEF) feeder layer, in the presence of leukemia inhibitory factor (LIF) to activate STAT3 and serum or bone morphogenetic protein (BMP) in order to inhibit differentiation and keep stemness of ESC. An alternative way to culture them is adaptation of ESC to feeder free condition. These cells resemble pluripotent features of epiblast cells in terms of unlimited self-renewal (maintenance of undifferentiated state) and differentiation toward three main embryonic lineages namely, endoderm, mesoderm and ectoderm (Evans and Kaufman 1981, Martin 1981, Niwa, Burdon et al. 1998, Ying, Nichols et al. 2003). The use of ESC is of great interest since they are immortal cells, which can proliferate infinitely with high speed.

In 2008 Austin Smith group showed that inhibition of differentiation inducing signaling from mitogen-activated protein kinase/ERK kinase (MEK) and glycogen synthase kinase 3 (GSK3) in ESC culture are required to maintain their proper stemness and self-renewal properties. The sustained undifferentiated ESC state that is attained by using inhibitors of these two pathways (2i) in culture medium is called "ground state" which is more similar to *in vivo* pluripotent state (Sato, Meijer et al. 2004, Ying, Wray et al. 2008).

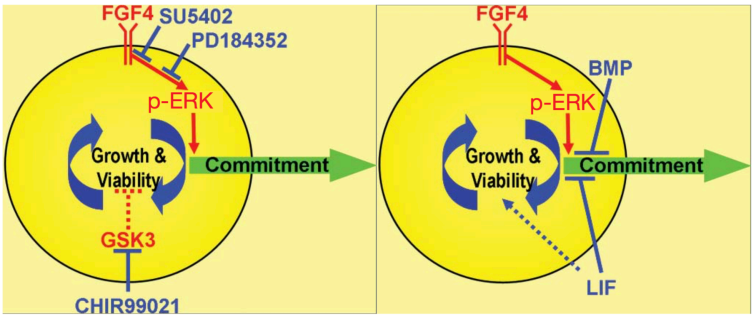


Fig 1.2. Inhibition of GSK3 serves a key function in augmenting self-renewal when phospho-ERK (p- ERK) is suppressed by maintaining cellular growth capacity.

Diagrams of self-replication of the pluripotent state when inductive phospho-ERK signaling is either inhibited upstream by chemical antagonists (left panel) or counteracted downstream by LIF and BMP (right panel). Adapted from (Ying, Wray et al. 2008).

4.1.2.2.Application

Due to infinite ability of ESC to self-renew but also their great potential of in vitro differentiation toward any desired epiblast-derived cell types (by providing the right signal transductions via defined growth factors at appropriate time), these cells could offer an endless pool of differentiated cell types. Thus, ESC could be considered as a robust and reliable physiological model for embryogenesis studies, drug discovery, patient-specific cell therapies and disease modeling in particular for diseases, affecting inaccessible cell types such as early embryonic stage cells or terminally differentiated cells, such as neural cells. That is why great efforts have been made in the past decade to establish the feasibility of using ESC for the above mentioned applications. However, despite substantial pros of using ESC over primary or immortalized cells, the usage of ESC are highly limited due to ethical debates following ESC derivation (Grskovic, Javaherian et al. 2011).

4.1.2.3.Somatic cell reprogramming to induced pluripotent stem cells (iPSC)

For the first time in 2006, the seminal work of *Shinya Yamanaka* showed that through ectopic expression of four transcription factors, namely: Oct4, Sox2, Klf4 and c-Myc, somatic cells can turn the developmental clock back and can be converted to embryonic stem cell-like state so called iPSCs *in vitro,* (Takahashi and Yamanaka 2006, Yamanaka 2012).

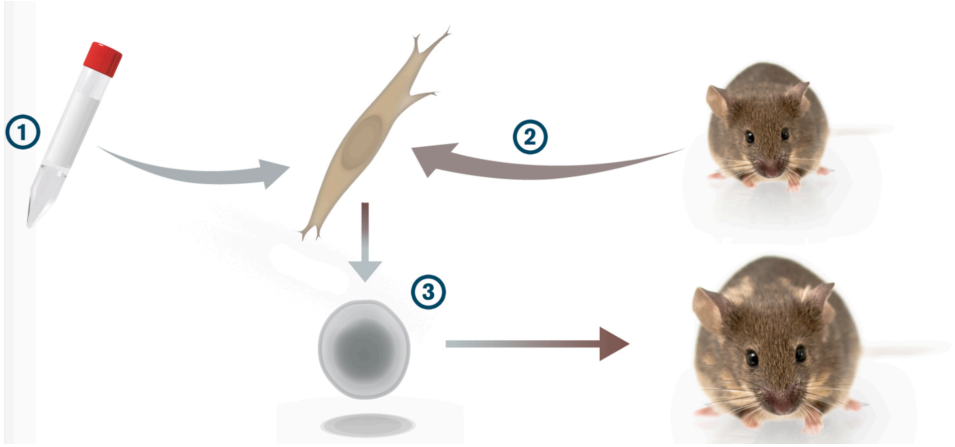


Fig 1.3. Somatic cell reprograming was achieved by forced expression of four key transcription factors.

Starting from a collection of 24 different transcription factors (symbolized by the test tube); (1), Takahashi and Yamanaka (2006) demonstrated that a set of only four transcription factors (Myc, Oct3/4, Sox2 and Klf4) was sufficient to convert cultured mouse embryonic or adult fibroblasts (2) to become pluripotent cells capable of producing teratomas in vivo and contributing to chimeric mice (3). The pluripotent cells were called induced pluripotent stem cells (iPS cells). Adapted from https://www.nobelprize.org/nobel_prizes/medicine/laureates/2012

This achievement was established based on three different streams of previous research. The first stream was generation of tadpoles upon nuclear transfer of intestinal cell nuclei from adult *Xenopus laevis* into unfertilized egg that was reported by John Gurdon in 1962 which is named somatic cell nuclear transfer (SCNT) (Gurdon 1962). Later *Ian Wilmut* consolidated this finding through generation of Dolly sheep, the first cloned mammal using the very same SCNT method upon nuclear transfer of mammary epithelial cells in to enucleated oocyte (Wilmut, Schnieke et al. 1997).

The second stream was identifying the inducing role of transcription factors in formation of myocytes from fibroblast and transformation of antennae into second legs in *Drosophila* (Davis, Weintraub et al. 1987, Schneuwly, Klemenz et al. 1987). And

ultimately, the third stream was the isolation and establishment of ESC in culture condition (Davis, Weintraub et al. 1987, Smith, Heath et al. 1988).

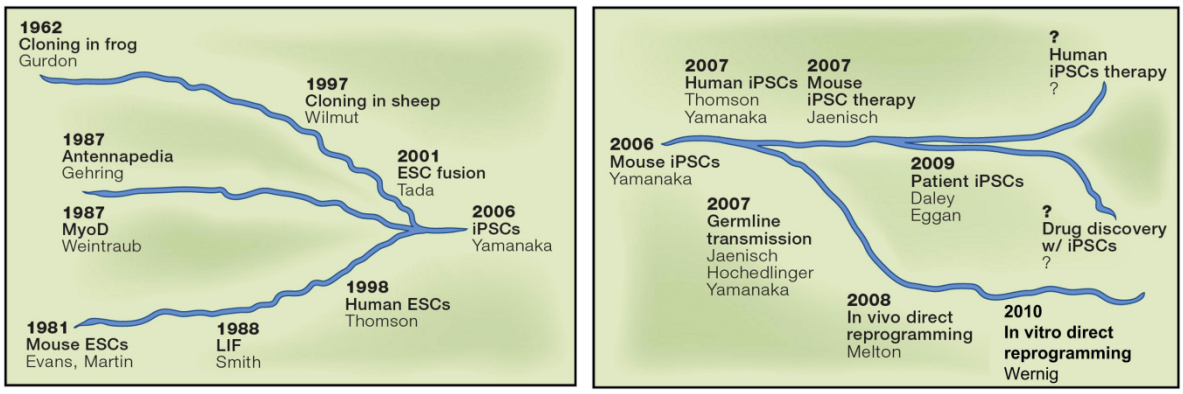


Fig. 1.4. Scientific streams led to or emerged from iPSC technology.

A) Three scientific streams that led to the development of iPSCs. B) New scientific streams that emerged from the development of iPSCs. Adapted from (Yamanaka 2012).

iPSC are not only comparable to ESC in terms of their morphology, transcriptional profile, epigenetic pattern and growth characteristics, but also, they can differentiate in vitro and in vivo to embryoid bodies and teratomas, respectively. Of note, similar to ESC, iPSC are capable of generating an entire mouse upon injection into the blastocyst of an embryo proving that they are indeed pluripotent (Takahashi and Yamanaka 2006). Therefore, this finding holds great promises for drug screening and regenerative therapies (Yamanaka 2012).

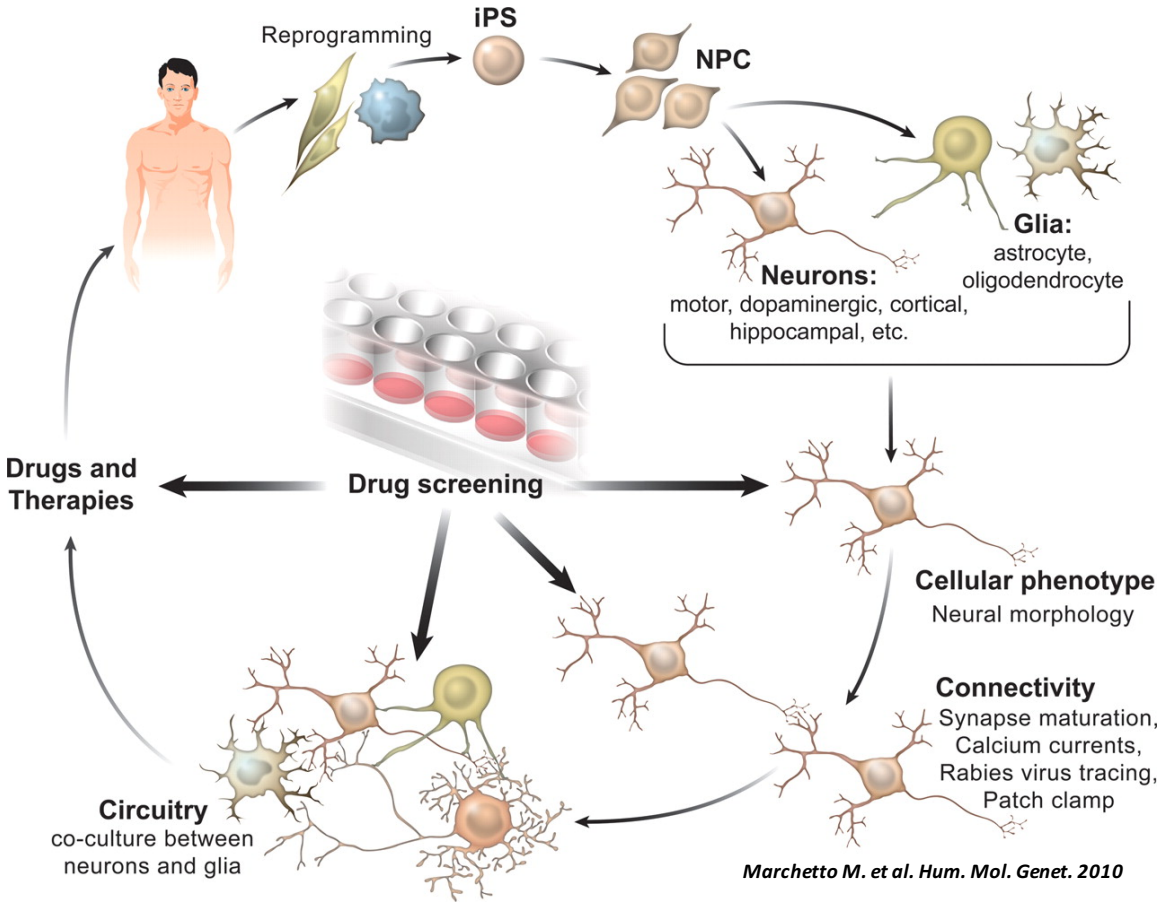


Fig 1.5. iPSC to model neurodegenerative and neurodevelopmental diseases.

Human iPSC from neurologic patients and controls are generated after somatic tissue reprogramming (e.g. skin or blood cells). Neural progenitor cells (NPC) are generated and are further differentiated into neurons and/or glial cells. Neurons are then differentiated into subtypes of neurons such as dopaminergic, cholinergic, etc. Cellular phenotype is assessed by measuring neuronal morphology (i.e. process branching, spine density/size/maturation). Next, connectivity and circuitry integration can be analyzed by calcium influx transients, electrophysiology and trans-neuronal tracing with the rabies virus. In addition, the cross-talk between neurons and glia can be studied to tease out autonomous and non-autonomous aspects of the disease. Once a distinct disease-related phenotype is identified, drug-screening platforms can be developed to test compounds that improve cellular phenotype. Therapeutic compounds could emerge from the screenings, potentially benefiting neurologic patients. Adapted from (Marchetto, Winner et al. 2010).

4.1.2.4. Limitation

However, in spite of several investigations on iPSC studies, the great passion of using these cells for regenerative medicine is confined due to inefficient reprogramming, lack of reproducible and robust differentiation protocols of iPSC and finally the genetic and epigenetic abnormalities of iPSC in culture that overall hinder the potential use of these cells for regenerative therapies and clinical applications (Vitale, Matigian et al. 2012). One of the key limitations of iPSC, is modeling diseases which occur at much primitive stage of embryonic development (prior to blastocyst stage). Similarly, the physiology of very early developmental stages cannot be studied via iPSC. Therefore, recently there is a great focus on establishment of cells with totipotnet-like features, which may present an additional opportunity for therapeutic purposes.

4.1.3. Totipotency

Totipotent cells (Zygote and its 2C blastomers) are the origin of all cells in the body since they are not only capable of differentiating in any cell type such as fetus embryonic three germ layers and all extra-embryonic tissues, but also, they can independently organize the developmental sequence to form a full organism without the need of carrier cells. (Macfarlan, Gifford et al. 2012, Condic 2014, Ishiuchi, Enriquez-Gasca et al. 2015, Zhou and Dean 2015). Of note, cells in later stages of embryonic development (e.g., 4-cell and 8-cell phases) are not considered any more totipotent since they are more restricted in their developmental potency.

Zygote has the minimal *de novo* transcript and it's mainly supported by the maternal storage. After the first cleavage, the transcription significantly increases in 2C-embryos that is called Zygote genome activation (ZGA). The newly synthesized ZGA

products progressively substitute the maternal storage to self-organize the early embryonic developmental events (Zhou and Dean 2015).

4.1.3.1.2C-like cells

Recent studies have identified 2C-like cells (also known as metastable state) as a rare transient sub-population of cells (~1-5%) within mouse embryonic (ESC) and induced pluripotent stem cell (iPSC) cultures that express high levels of transcripts also found in totipotent two-cell (2C) embryos (Zalzman, Falco et al. 2010, Macfarlan, Gifford et al. 2012, Akiyama, Xin et al. 2015). Notably, nearly all PSCs cycle in and out of this metastable state, at least once during nine passages that is also accompanied by the fluctuating expression of some key 2C genes such as Zscan4 gene family and transient transcriptional activation of endogenous retroviruses (ERVs), in particular MuERV-L.

4.1.3.2.Zscan4

Zscan4 (zinc finger and SCAN domain containing 4) gene cluster in mouse accommodates six highly similar transcribed paralogous genes (Zscan4a–f) and three pseudogenes that are accordingly called Zscan4 (Falco, Lee et al. 2007). During pre-implantation development Zscsn4 is exclusively expressed in 2C stage as it is not detectable in later stages and in blastocysts, but when blastocysts are cultured as an outgrowth, a few percentages of ICM derived ESC begin to express Zscan4. Remarkably, knocking down Zscan4 in two-cell embryos avoids the implantation to uterine wall due to one-day delay that occurs during embryonic development. Of note, Zscan4d is predominantly expressed in 2C-embryos while Zscan4c is mostly expressed in about 1-5% of ESC culture (Falco, Lee et al. 2007, Ko 2016).

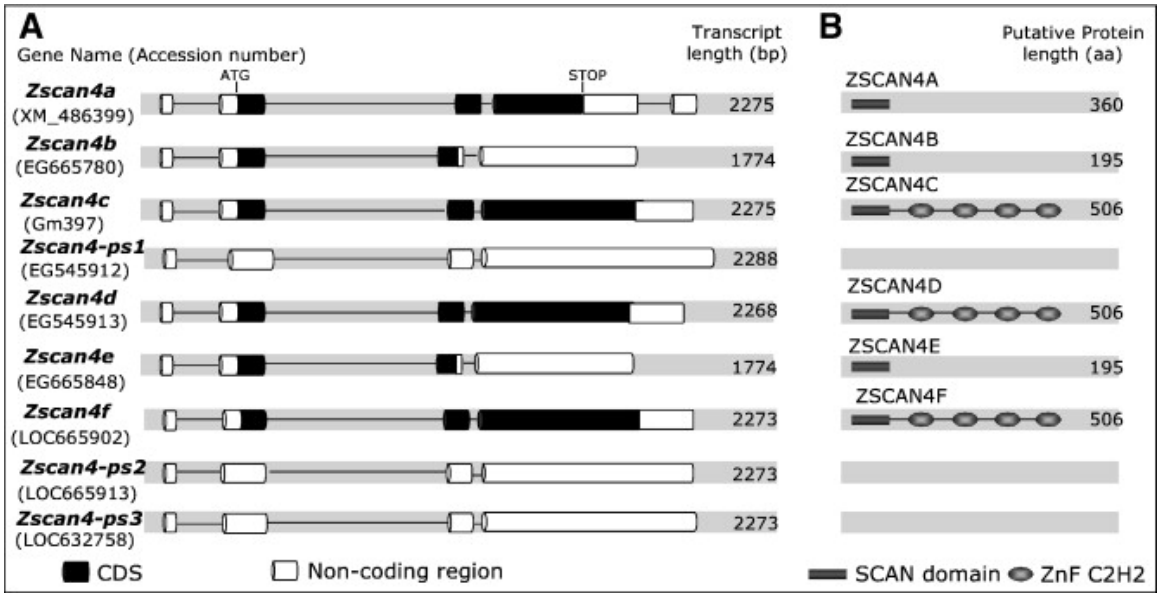


Fig 1.6 The Zscan4 family.

(A) Exon–intron structures of nine Zscan4 paralogs. New gene symbols proposed are shown in bold italics with the current gene symbols. (B) Putative protein structures of Zscan4 paralogs. Adapted from (Falco, Lee et al. 2007).

Interestingly, the remarkable genomic stability of mouse pluripotent stem cells has been recently linked to the transient bursts of Zscan4 expression during this metastable state. Zscan4 knockdown in PSCs shortens telomeres, increases karyotype abnormalities and spontaneous sister chromatid exchanges that subsequently lead to crisis by passage eight. On the other hand, transient overexpression of Zscan4 promotes telomere sister chromatid exchange (T-SCE) and telomere recombination as it causes telomerase-independent extension of telomere length in mouse ESC and decreases spontaneous SCE throughout the genome (Zalzman, Falco et al. 2010). These results overall suggest that transition to 2C-like state in PSC culture could be beneficial for proper proliferation and

also to maintain the genomic stability of these cells (Zalzman, Falco et al. 2010, Amano, Hirata et al. 2013).

Though ESC are capable of maintaining their potential to generate an entire animal upon several passages in culture, it is known that the long-term culture may drop the efficiency of this process (Suda, Suzuki et al. 1987). Importantly, it has been demonstrated that ESC could sustain their high potency of chimeric mouse generation through frequent activation pulses of Zscan4, even in long-term culture conditions. Moreover, it has been found that sorted Zscan4^{+} cells have lower developmental potency with respect to Zscan4^{-} population suggesting that although Zscan4^{+} state itself, lacks developmental potency, the reversible transition to Zscan4^{+} state is needed to rejuvenate ESC and recover their developmental potency (Amano, Jeffries et al. 2015). Of note, it is demonstrated that constitutive expression of Zscan4 leads to slowing down or blocking of proliferation in both MEF and ESC (Hirata, Amano et al. 2012).

Furthermore, it is revealed that overexpression of Zscan4 together with conventional reprogramming factors not only could increase the somatic cell reprogramming efficiency, but also enhance the quality of formed iPSC as shown by tetraploid complementation assay. Importantly, Zscan4 expression, which transiently reactivates pre-implantation specific genes, is only needed for the first few days of reprogramming, while other reprogramming factors are not dispensable during entire reprogramming process (Hirata, Amano et al. 2012, Jiang, Lv et al. 2013).

A recent study revealed that during transition from Zscan4^{-} to Zscan4^{+} and *vice versa* (Zscan4-associated event; Z4 event) dynamic epigenetic changes arise in ESC, i.e. derepression of heterochromatin in Zscan4^{+} followed by rerepression upon switching to Zscan4^{-} cells. Derepression of chromatin is accompanied by DNA demetylation, histone

acetylation (for instance H3K27ac) and a burst of transcription from typically silenced regions of the genome. This heterochromatin-based transcriptional activity suggests that mESCs may maintain their extraordinary genome stability at least in part by transiently resetting their heterochromatin (Akiyama, Xin et al. 2015). Several studies proved that in cancer cells the expression of heterochromatin genes e.g. retrotransposons through opening of heterochromatin regions of the genome and massive transcription from these sites threaten genomic integrity of cells and are dangerous for pre implantation development However, it is speculated that early embryos encounter this treat by transient blocking of global protein synthesis (please see section 4.1.3.3). (Egger, Liang et al. 2004, Ting, Lipson et al. 2011, Carone and Lawrence 2013, Ko 2016).

Strikingly, two recent studies demonstrated that in *Drosophila* and *Caenorhabditis elegans,* substantial transcriptional activation during ZGA triggers intrinsic DNA damage and since keeping the genome integrity at very early stage of development is very critical for the embryo, having the capability of accurate repair mechanisms is of paramount value to avoid the transmission of mutations to the entire body or even next generation.

Therefore, it is reasonable to think that Zscan4 and other 2C related genes like Tcstv1&3 are possibly activated to improve the genome stability of early embryonic cells. In particular, Zscan4 may increase homologous-recombination mediated-repair, which is the most accurate and precise repair mechanism that exists in germ cells as it has been shown that in Zscan4 positive ESC, meiosis-specific homologous recombination genes are upregulated (Tichy, Pillai et al. 2010, Zalzman, Falco et al. 2010, Blythe and Wieschaus 2015, Butuci, Williams et al. 2015, Ko 2016).

Aneuploidy of human pre-implantation embryos is an important concept that needs to be considered for failure of appropriate development of an embryo. However, several

works have recently reported healthy newborns despite aneuploidy diagnosis at much primitive stages of development possibly due to either selection or self-corrections mechanisms present in preimplantation embryos. Importantly, *in vitro* fertilization studies represent that karyotype abnormalities after ZGA in human embryos (day5) are much less than at day3 where ZGA has not occurred yet, suggesting that some ZGA-specific repair mechanisms might be involved in self correction of chromosomal abnormalities (Bazrgar, Gourabi et al. 2013, Ko 2016). Interestingly, Ko lab showed that not only transient overexpression of Zscan4 (one of the key ZGA genes) in mouse ESC improves their karyotypic features, but also it can correct karyotype abnormalities of human primary fibroblast cells derived from Down syndrome (Trisomy 21) and Edwards syndrome (Trisomy 18) patients (no mechanistic evidences on how this could happen is discussed in this paper) (Zalzman, Falco et al. 2010, Amano, Hirata et al. 2013, Amano, Jeffries et al. 2015, Ko 2016). These evidences overall suggest that activation of Zscan4 and possibly some other two cell specific genes are important for the proper development of embryos .

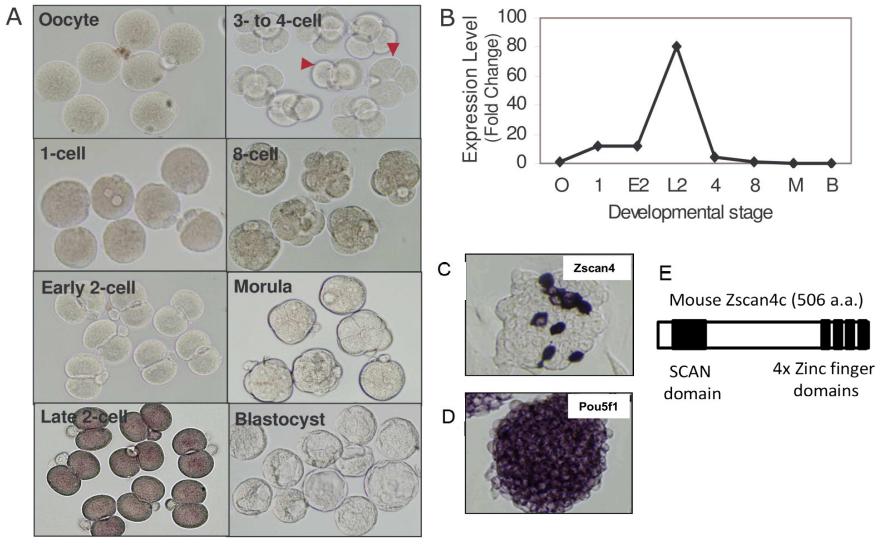


Fig 1.7. Expression profiles of Zscan4 in mouse preimplantation embryos and mouse ES cells.

(A) Whole-mount RNA in situ hybridization of mouse preimplantation embryos with a Zscan4 probe. (B) qRT-PCR analyses of Zscan4 expression during preimplantation development. (C) Whole-mount RNA in situ hybridization of mouse ES cell colonies with a Zscan4 probe. (D) As a control, whole-mount RNA in situ hybridization with Pou5fa (a.k.a. Oct3/4 or Oct4). (E) Structure of mouse Zscan4 protein (Zscan4c). Modified from (Falco, Lee et al. 2007, Ko 2016).

4.1.3.3.Eif1a-like genes

Eukaryotic translation initiation factor1A (Eif1a) is one of ZGA-specific genes that are transiently expressed in 2C embryos. However, recent studies showed that it is not EiF1a itself, but Eif1a-like genes, which are expressed in Zscan4^{+} cells and 2C-embryos. These genes may act as a dominant negative competitor of Eif1a, since their over expression could repress the global protein synthesis in mouse ESC during Z4 event. In particular it has been suggested that the expression of Eif1a-like genes could block the translation of the harmful genes to keep the genomic integrity of mouse ESC (Davis, DeSousa et al. 1996, Hung, Wong et al. 2013, Ko 2016).

4.1.3.4.Tcstv1 and Tcstv3

Tcstv1 (two-cell-stage, variable group, member 1) and Tcstv3 (two-cell-stage, variable group, member 3) are other two originally reported 2C-stage transcripts that when overexpressed, increase the expression of Zscan4 and regulate the length of telomeres. Therefore, they play an essential role in long-term maintenance of ESC (Zhang, Dan et al. 2016).

4.1.3.5.MuERV-L

Murine endogenous retrovirus-like gene (MuERV-L) is highly abundant mRNA at the 2-cell stage of development but it undergoes repression through various epigenetic

silencing mechanisms at the blastocyst stage. Such a tight regulation is essential to keep the balance between destructive outcomes of retro-transpositions in the genome versus the benefits of promoting genetic diversity. Recent data indicate that MuERV-L regulates the unique transcriptional signature of both 2C-like cells and 2C-embryos through various mechanisms including; the activation of enhancers, introducing alternative promoters and generation of chimeric transcripts for early embryonic mouse genes. (Macfarlan, Gifford et al. 2011, Macfarlan, Gifford et al. 2012, Schoorlemmer, Perez-Palacios et al. 2014, Robbez-Masson and Rowe 2015, Zhou and Dean 2015). Besides this, MuERV-L positive 2C-like PSC, are found to have more expanded developmental capacity, as they contribute to extra-embryonic tissues such as yolk sac, TE and placenta, in contrast to normal PSC which can only contribute to embryonic lineages (Macfarlan, Gifford et al. 2012).

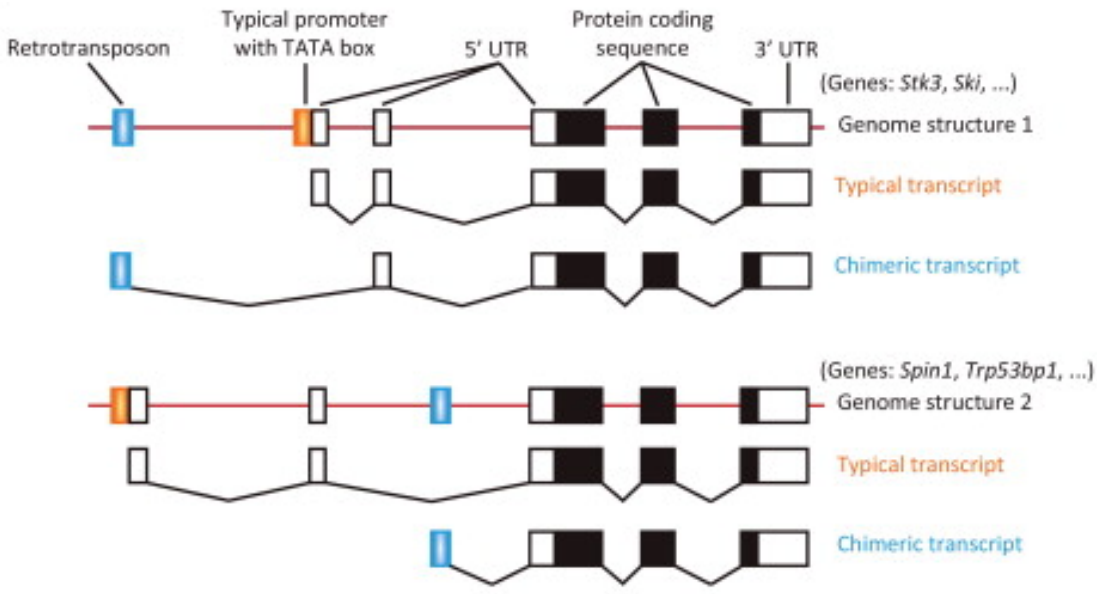


Fig 1.8. Retrotransposons are activated during the preimplantation stage

and provide alternative first exons to activate downstream embryonic genes, producing chimeric transcripts that are absent in other cell types. Adapted from (Zhou and Dean 2015).

4.1.3.6. Regulation of 2C-like cells in PSC culture

Several genetic and culture related alterations have been shown to regulate the population of 2C like cells in ESC and iPSC culture. Here I summarize each study highlighting their main findings:

4.1.3.6.1. Environmental factors:

Growing ESCs in 2i media (containing 3mM glycogen synthase kinase 3b (GSK3b) and mitogen-activated protein-kinase kinase (MEK) inhibitors) that is known as 'ground-state' media condition drops the percentage of 2C-like cells in ESC culture in comparison to knock out serum (KOSR) or fetal calf serum (FCS) containing media. Moreover, culturing ESCs in 20% oxygen (O_2) tension also has been found to increase the 2C-like population in comparison to 5% O_2 culture condition, suggesting some extrinsic and intrinsic regulatory mechanisms for expression of 2C specific genes in PSC culture. (Macfarlan, Gifford et al. 2012).

4.1.3.6.2. Genetic factors

Few studies attempted to identify key molecular players, linked to the transition from pluripotent to totipotent-like state. For instance, previous reports demonstrated that 2C-specific genes were increased in mutant ES cells lacking the histone lysine-specific demethylase gene Kdm1a (also known as LSD1), chromatin assembly factor-1 (CAF-1), the H3K9 histone methyltranferase G9a, KRAB (Kruppel- associated box)-associated transcriptional repressor Kap1 and ten-eleven translocation (TET) proteins that will be discussed briefly below. (Macfarlan, Gifford et al. 2012, Maksakova, Thompson et al. 2013, Rowe, Friedli et al. 2013, Lu, Liu et al. 2014, Ishiuchi, Enriquez-Gasca et al. 2015).

4.1.3.6.2.1.KAP1

KAP1 is a member of the RBCC (ring, B-box, coiled–coiled) or TRIM (tripartite motif) family of proteins. It is recruited to genes by the tetrapod- specific, DNA sequence-specific KRAB-ZFPs (Kruppel-associated box domain-zinc finger proteins) in vertebrates. Due to the fact that KAP1-mediated repression could contribute to the control of ERVs and that KAP1 can trigger permanent gene silencing during early embryogenesis, Rowe et al., aimed to identify KAP1 target genes and their KRAB zinc finger intermediates.

Interestingly, they found that KAP1 deletion leads to a marked upregulation of a range of ERVs, in particular IAP elements, in mouse embryonic stem cells but also in early embryos. Importantly, this was associated with loss of histone 3 lysine 9 trimethylation (H3K9me3), a hallmark of KAP1-mediated repression. These results overall demonstrate that KAP1 controls endogenous retroviruses in mouse embryonic stem cells (Rowe, Friedli et al. 2013).

4.1.3.6.2.2.CAF-1

In a recent study from Torres-Padilla lab, authors aimed to identify molecular players associated with transitions between pluripotent and totipotent-like states. Interestingly, they found that depletion of either the p150 or the p60 subunits of chromatin assembly factor-1 (CAF-1) in mouse ES cells increases the population of 2C-like-cells which was accompanied by the increased accessibility at MuERV-L, leading to the upregulation of neighboring genes. Moreover, they show that induced 2C-like cells after CAF-1 depletion display a transcriptional program and the chromatin features of 2-cell-stage embryos.

These results along with the higher reprogrammability of CAF-1 depleted ESCs through SCNT experiment, overall show that mouse 2C-like cells can be induced *in vitro*

through downregulation of the chromatin-assembly activity of CAF-1. (Ishiuchi, Enriquez-Gasca et al. 2015)

4.1.3.6.2.3.KDM1A

In a similar attempt, the elevation of MuERV-L and 2C-specific genes in KDM1A mutant ES is well documented. KDM1A (LSD1) is the first lysine-specific demethylase 1 that is identified, which demethylates histone H3 mono- and dimethyl K4 and H3K9 and depending on the affected histone residue, it either boosts or suppresses gene expression. Genome-wide epigenomic profiling of KDM1A mutant ESC demonstrated that in the absence of KDM1A, in MuERV-L and a set of 2C- specific genes some chromatin modifications occur that favors the derepression of the chromatin, including hyper-methylation of histone H3K4, hyper-acetylation of H3K27, and hypo-methylation of H3K9 suggesting that KDM1A is crucial for repressing the expression of genes during early phases of embryo development (Macfarlan, Gifford et al. 2011).

4.1.3.6.2.4. TET family

Ten- eleven translocation (Tet) family protein are known to be involved in the active DNA demethylation through oxidizing 5mC to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al. 2009; Ito et al. 2010), which in turn can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) followed by base excision repair to complete the demethylation cycle (He, Li et al. 2011, Ito, Shen et al. 2011, Shen, Wu et al. 2013).

As the functional characterization of Tet proteins has been complicated by the redundancy between the three family members, Lu and Liu et al., generated mouse embryonic stem cells (ESCs) deficient for all three Tet proteins (Tet triple knockout [TKO]) using CRISPR/Cas9 technology. Importantly they reported a similar transcriptional feature of 2C-like state in Tet TKO ESCs. Furthermore, they found that Tet

TKO ESCs exhibited increased telomere-sister chromatid exchange and elongated telomeres in line with previous report highlighting a remarkable feature of 2C-like cells in extending their telomeres (Lu, Liu et al. 2014).

4.1.3.7.Application

Although the direct reprogramming of differentiated cells to *bona fide* totipotent (like) state is yet too far, uncovering how 2C-like genes are reactivated can give insights on the physiology of early embryonic development and may give some clue to make this process of reprogramming to totipotent cells feasible. Here I summarize the potential applications of 2C-like cells.

4.1.3.7.1. Disease modeling for diseases develop at the primitive stage of embryonic development

As previously mentioned, one of the key applications of iPSC is disease modeling, however, iPSC cannot model disease which could initiate at much primitive stages of development (prior to blastocyst formation). For example, totipotent-like cells could be an ideal recourse for large group of disease known as placenta disease.

4.1.3.7.2. Study of early stages of embryonic development

One of the main reasons why our knowledge about the physiology and unique mechanisms activated at early embryonic stage is limited is due to the fact that we do not have faithful *in-vitro* model which can recapitulate these stages. Thus, totipotent stem cell could be instrumental to study the physiology and molecular mechanism underlying these early stages.

4.1.3.7.3. Improving genomic stability of PSC

It is known that iPS cells are prone to gain genomic instability during the reprogramming process but also over the several steps of propagation in culture. This could dramatically hinder their application in disease modeling and regenerative medicine (Grskovic, Javaherian et al. 2011). As discussed previously, one of the mechanism that PSC could exploit in culture to tackle this problem is through undergoing metastable stage to activate totipotency genes, which are proficient in repairing damaged DNA. It is shown that not only down-regulation of these genes leads to genomic instability and karyotype abnormalities of ESC but more importantly their transient over-expression could lead to telomere elongation (Zalzman, Falco et al. 2010) Overall, these findings demonstrate that the totipotent cells are more proficient in DNA repair mechanisms and that the transient conversion to totipotent state could be beneficial to improve the genomic stability of PSC. Thus, understanding how DNA is repaired at the early stages of mammalian embryonic development could be of great importance.

4.1.3.7.4. Higher efficiency of reprogramming

Somatic cell nuclear transfer (SCNT) is a method to generate a viable embryo during which a donor nucleus from a somatic cell is implanted in enucleated oocyte. SCNT is used in both therapeutic and reproductive cloning, for examples, Dolly the Sheep became famous for being the first successful case of the reproductive cloning of a mammal. "Therapeutic cloning" refers to the potential use of SCNT in regenerative medicine as this approach has been championed as an answer to the many issues concerning embryonic stem cells (ESC) and the destruction of viable embryos for medical use. One of the main limitations of SCNT is that it is incredibly inefficient (Wilmut,

Schnieke et al. 1997). Interestingly, recent papers show that totipotent-like cells have much higher capacity for SCNT rather than pluripotent stem cell thus conversion to totipotent state could be considered as an alternative way to improve the efficiency of SCNT (Ishiuchi, Enriquez-Gasca et al. 2015). Furthermore, 2C-like specific genes could be utilized for increasing the efficiency of somatic cell reprogramming as it has been shown that by overexpression of Zscan4 the efficiency of reprograming has increased significantly (Hirata, Amano et al. 2012).

4.2. DNA replication and replication stress

4.2.1. Cell Cycle

Cell cycle is the cellular life cycle that through sequential series of events cell regulates its precise DNA replication and cell division. The cell cycle of eukaryotic cells consists of four discrete phases namely G1, S, G2 and M phase. Genomic DNA is replicated during specific synthesis phase (S) of the cell cycle, which is distinctly separated by gap-phase two (G2) from mitosis (M) where segregation of duplicated DNA to daughter cells occurs. Mitosis is followed by cytokinesis that splits the cytoplasm between to newly generated daughter cells. Two gap phases between M-phase and S-phase (namely G1) and between S-phase and mitosis (i.e., G2) are essential not only for duplication of the required components of the cell, but also to ensure that the conditions are favorable for accurate DNA replication in each cycle through series of checkpoints (Hartwell and Weinert 1989).

4.2.2. Eukaryotic DNA replication

DNA replication is a semi-conservative mechanism to preserve the genome through accurate duplication of DNA strictly once during each cell cycle. In eukaryotes, DNA

replication starts in parallel from several predefined sequences so-called replication origins that are spread across the whole genome. During the late M transition to early G1 phase of the cell cycle, origin recognition protein (ORC) recognizes the origins, and then Cdt1 and Cdc6 bind to ORC to create pre-replicative complex (preRC) that is essential for the helicase (MCM2-7 complex) loading onto DNA (This step is referred to replication licensing). In the next step (G1-S transition), the key replication initiation proteins such as Cdc45 and GINS complex are recruited to the DNA through the activation of cycline dependent kinase (CDK) and Dbf4 dependent Cdc7 kinases in order to activate replication fork and trigger DNA replication initiation. Of note, only a subset of licensed origins are activated in each cell cycle while others remain dormant (Yekezare, Gomez-Gonzalez et al. 2013) Firing of inactive (dormant) origins plays an essential role in maintaining the integrity of the genome in case of fork stalling and replication stress (Yekezare, Gomez-Gonzalez et al. 2013). Therefore, the organized control of origin firing is fundamental for a single round of accurate and timely replication of the genome in each cell cycle (Masai, Matsumoto et al. 2010). Upon firing of licensed origins, DNA replication proteins construct a fork shape structure, called replication fork (RF) where key replication factors coordinate the replication progress. Briefly, helicase activity of MCM complex unwinds the DNA duplex; and RPA protein stabilizes the generated ssDNA, follwing which the replication elongation takes place via DNA polymerases (pol ε and pol δ) on leading and lagging strands of the DNA, respectively (Langston, Indiani et al. 2009, Masai, Matsumoto et al. 2010).

4.2.3. DNA damage response (DDR)

Maintenance of the DNA integrity is of paramount value to guarantee the protection of genetic information in all living organisms. Thus, to encounter several kinds of genotoxic stress that organisms are exposed to, particular mechanisms have been evolved. Depending on the type of the DNA damage, DDR pathways is determined by activation of different members of PI3K-like kinases (PIKKs) family, i.e. ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Sulli, Di Micco et al. 2012).

ATM is mainly activated in response to double strand breaks (DSB), while ATR is mostly involved in detection of ssDNA. Both kinases phosphorylate the histone variant H2AX on Ser139 (γH2AX) in the close region to the DNA lesion. Eventually, ATM and ATR in turn phosphorylate Chk2 and Chk1 kinases, respectively, in order to diffuse the signal and subsequently activate downstream effector (P53 and cell division cycle 25 (CDC25)). Activation of effectors end up in various consequences including transient cell cycle arrest and restarting of proliferation after DNA repair, programmed cell death (apoptosis) or senescence (Sulli, Di Micco et al. 2012). Interestingly, several studies have shown that DNA damage in ESC and adult stem cells induce differentiation as an alternative outcome of DDR activation (Lin, Chao et al. 2005, Tichy and Stambrook 2008, Tichy, Pillai et al. 2010, Serrano, Liang et al. 2011, Schneider, Pellegatta et al. 2013, Santos, Faryabi et al. 2014).

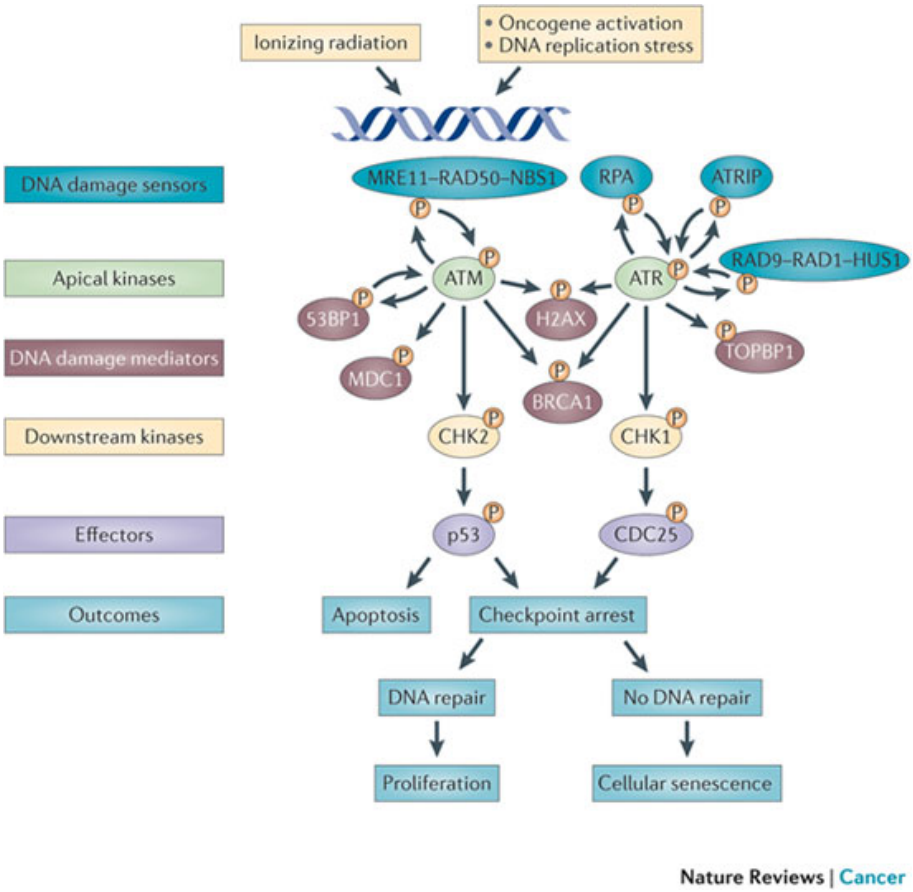


Fig 1.9. DNA damage response.

DDR pathway is composed of two main DNA damage sensors: the MRE11–RAD50–NBS1 (MRN) complex that detects DNA double-strand breaks (DSBs); and replication protein A (RPA) and the RAD9–RAD1–HUS1 complex that detects exposed regions of single-stranded DNA. These sensors recruit the apical kinases ATM (through the MRN complex) ATR (through RPA and the 9-1-1 complex, which is bound by ATR-interacting protein (ATRIP). These in turn phosphorylate (P) the histone variant H2AX on Ser139 (known as γH2AX) in the region proximal to the DNA lesion. Eventually, DDR signaling spreads away from the damaged locus owing to the engagement of diffusible kinases CHK2 (which is mainly phosphorylated by ATM) and CHK1 (which is mainly phosphorylated by ATR). DDR-mediated cellular outcomes may be cell death by apoptosis; transient cell cycle arrest followed by repair of DNA damage and resumption of proliferation; or cellular senescence caused by the persistence of unrepaired DNA damage. Adapted from (Sulli, Di Micco et al. 2012).

4.2.4. Replication stress (RS)

Although there is no single universally-accepted definition for replication stress, usually slowing down the DNA synthesis or stalling of replication fork progression is referred as “replication stress” that usually occurs when helicase continue to unwind the parental DNA even after polymerase stalling and results in formation of the aberrant fork structures with single-stranded DNA (ssDNA) stretches (Pacek and Walter 2004, Magdalou, Lopez et al. 2014, Zeman and Cimprich 2014).

4.2.4.1.Sources of replication stress

Many intrinsic and extrinsic obstacles may challenge the appropriate completion of DNA replication, which can cause replication stress. Nicks and stretches of ssDNA, barriers for replicative helicase or polymerase, unrepaired DNA lesions, collisions between replication and transcription machinery, constitutive activation of oncogenes and physical barriers on the chromatin such as secondary DNA structures or DNA-protein complexes are some of the possible factors which could trigger replication stress. (Magdalou, Lopez et al. 2014, Zeman and Cimprich 2014). Moreover, aphidicolin “a specific inhibitor of DNA polymerase ε and DNA polymerase δ “ is a widely used treatment, which disrupts correct DNA replication and induces replication stress (Krokan, Wist et al. 1981, Baranovskiy, Babayeva et al. 2014).

4.2.4.1.1. Altered origin firing activity

Altered origin firing activity is another important factor, which can induce replication stress. For instance, over-licensing of origins (firing too many origins) may deplete the dNTP pool and slow down the fork progression. On the other hand, firing too

few origins can lead to replication stress as well, since it makes it difficult for the replication forks to travel long distances on the genome and it causes under-replication which leads to loss of genetic information in particular in regions that are hard to be replicated such as heterochromatins . (Letessier, Millot et al. 2011, Magdalou, Lopez et al. 2014, Zeman and Cimprich 2014). In addition, it has been shown that Cdc45, a key factor required for replication initiation, is rate limiting for origin firing in mammalian cells since it is present at much lower concentration with respect to other replication factors suggesting that the overexpression of Cdc45 could activate dormant origins and increase the overall replication rate (Wong, Winter et al. 2011). Besides, it has been proved that, similar to c-Myc, in *Xenopus,* Cdc45 overexpression induces replication stress by firing too many origins that subsequently leads to DNA damage (Srinivasan, Dominguez-Sola et al. 2013).

4.2.4.1.2. Limited source of nucleotides

The amount of nucleotides needs to be well-balanced for the successful accomplishment of replication process as deregulation of nucleotide pool may result in replication stress, mutagenesis and genomic instability. In over-activated origins, replication stress is induced due to quick exhaustion of the existing dNTP pool. Moreover, hydroxyurea (HU), which inhibits ribonucleotide reductase enzyme, depletes nucleotide storage of the cells and induces replication stress suggesting that supplying the additional amount of nucleotides through exogenous sources may rescue the fork stalling and replication stress (Young and Hodas 1964, Poli, Tsaponina et al. 2012).

4.2.4.2.Replication stress response

As it is mentioned above, replication stress mainly take place due to the helicase-polymerase uncoupling, accompanied by accumulation of ssDNA in the close district to stalled replication fork and progressed by the recruitment of replication protein A (RPA) to ssDNA. It has been well-documented that RPA-coated ssDNA serves as a signaling platform for mediating replication stress response and recruiting the key replication stress-proteins such as ataxia-telangiectasia mutated (ATM)- and Rad3-related (ATR) kinases. (Pacek and Walter 2004, Byun, Pacek et al. 2005, Zeman and Cimprich 2014). Activation of ATR as the "central replication-stress-response kinase" phosphorylates its substrates to help cells to complete their accurate replication in stressing conditions. Phosphorylation of the histone variant H2AX (γH2AX) is one of the most commonly used markers to detect replication stress. However, since γH2AX can be detected in different types of DNA damage, it is not a specific replication stress marker. Though, phosphorylation of RPA (on Ser 33) or CHK1 (on Ser 345), detection of ssDNA directly or indirectly through native BrdU immunofluorescence or RPA foci formation, measurement of DNA synthesis progress through DNA combing assay, detection of higher levels of ssDNA gaps and detecting reversed forks through electron microscopy approach could more specifically reflect ATR dependent replication stress (Zou and Elledge 2003, MacDougall, Byun et al. 2007, Nam and Cortez 2011, Bianco, Poli et al. 2012, Marechal and Zou 2013, Ahuja, Jodkowska et al. 2016).

Upon replication stress, ATR helps to stabilize and restart the stalled fork after the source of replication stress has been removed. However, if the source cannot be banned, two other mechanisms prevent prolongation of fork stalling and restart the replication fork. The first mechanism is firing close by dormant origins (Yekezare, Gomez-Gonzalez et al. 2013) and the second one is continuing the replication downstream of the stalled fork by

leaving the un-replicated ssDNA gap close to the lesion in order to be replicated later through compensatory mechanisms such as DNA damage tolerance pathway (DTT). For example, cells can bear the presence of un-replicated ssDNA gaps during replication through utilizing specialized polymerases or sister chromatid as a template (Lopes, Foiani et al. 2006, Petermann and Helleday 2010, Elvers, Johansson et al. 2011, Mailand, Gibbs-Seymour et al. 2013).

Nevertheless, in spite of such complex mechanisms that cells exploit to keep the stalled fork stable, replication forks could collapse or become inactive as a result of persistent replication stressing source or loss of ATR response pathway components which in turn lead to double strand break (DSB) formation and activation of other DNA damage response markers i.e. ATM and DNA-PK. (Lopes, Cotta-Ramusino et al. 2001, Tercero and Diffley 2001, Chanoux, Yin et al. 2009) (Ciccia and Elledge 2010).

4.2.4.3.Replication stress and human diseases

Several human diseases are directly associated with deficiencies in the replication stress response pathways. Fork collapse, deficient DNA replication and deregulation of transcription or other processes linked to DNA replication and the way cells cope with stresses, all together could potentially contribute to DNA damage, mutations and eventually diseases. Depending on the proteins and replication stress signaling pathways that are defected, there are huge varieties in disease phenotypes. The name and associated phenotype of each disease plus affected DNA response proteins are listed precisely in (Zeman and Cimprich 2014) review.

4.2.4.4. ATR Seckel syndrome

ATR is the central kinase to be activated during replication stress and its total loss is shown to be embryonic lethal. Furthermore, drop in ATR protein expression in human patients and animals with a hypomorphic allele of ATR or a mutation in ATR-Interacting Protein (ATRIP) that is required for ATR stability and recruitment to the site of damage, results in a severe syndrome called Seckel syndrome. These patients suffer from microcephaly, developmental delay and mental retardation.

It has been demonstrated that conditional knock down of ATR is sufficient to accelerate premature aging related phenotypes (Ruzankina, Pinzon-Guzman et al. 2007). A mouse model of Seckel syndrome has been generated by Fernandez-Capetillo group in 2009 by swapping the mouse ATR allele with the human counterpart and introducing the Seckel mutation from A to G in exone 9. This mutation promotes the skipping of exone 9 during splicing and results in severe ATR hypomorphism. Interestingly, Capetillo group reported high levels of replicative stress in ATR Seckel mice mostly during embryogenesis, when proliferation is widespread. However, replicating stress was reduced to marginal amounts in postnatal life. Moreover, adult Seckel mice showed accelerated aging recapitulating of Seckel syndrome despite of decrease in replication stress in postnatal life. Altogether, these results suggest that replicative stress, particularly in utero, could contribute to the onset of aging in postnatal life (O'Driscoll and Jeggo 2008, Murga, Bunting et al. 2009, Ogi, Walker et al. 2012, Zeman and Cimprich 2014).

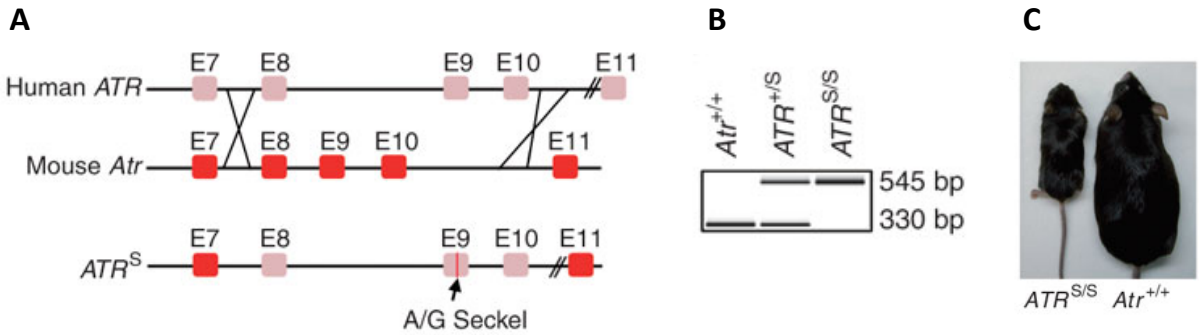


Fig 1.10. Strategy for generating the mutant ATR allele.

A) The rearranged allele contains the genomic region encompassing human exons (E) 8–10 (pink) inserted into the equivalent region of the mouse ATR gene (red exons). The Seckel syndrome mutation is indicated in E9. B) Analysis of littermate MEF lines by PCR genotyping (RT-PCR of ATR with primers at E8 and E10). C) Representative pictures of ATR+/+ and ATRS/S mice at 3 months of age. Modified from (Murga, Bunting et al. 2009)

4.3. Cell cycle and DNA damage response in ESC

4.3.1. Cell cycle profile of pluripotent cells

Mouse ESC have a particular and rapid cell cycle profile which takes about 10-12 hours with respect to somatic cells in which the cell cycle length is more than 24 hrs. (Stead, White et al. 2002)

The unique cell cycle profile of ESC features a high proportion of cells in S-phase (60-70%) and very short G1 phase with a smaller proportion of cells (15-20%) in comparison to somatic cells that spend most of their time (more than 70%) in gap phases and less time in S-phase (about 20%). Nevertheless, the lengths of the S-phase and M phase in pluripotent cells are comparable to somatic ones (White and Dalton 2005, Li, Ballabeni et al. 2012, Suvorova, Katolikova et al. 2012).

Furthermore, ESC not only have much higher amount of cell cycle regulatory factors such as Cdc6, Cdc25a and cyclins in comparison to MEFs, but also, most of these regulatory factors in particular the ones monitoring the proper origin licensing, remain constant. This is in contrast to somatic cells where such regulatory elements are oscillating (degraded and resynthesized) during each cell cycle (Tichy, Pillai et al. 2012).

Of note, ESC have a compromised G1-S checkpoint (cell cycle arrest) following DNA damage, which is considered as a safeguard mechanism to prevent differentiation, since it has been shown that in G1 phase, MEK signaling pathway regulates the transcription of genes responsible for induction of early differentiation. Moreover, the suppression of MEK pathways negatively regulates the proliferation of somatic cells; while it doesn't affect ESC growth and even contributes to keep ESC undifferentiated. (Ying, Wray et al. 2008, Suvorova, Katolikova et al. 2012).

Above-mentioned remarks, could explain the higher proliferation rate in pluripotent cells and their peculiar cell cycle profile which is needed to maintain their self-renewal and pluripotency features (Suvorova, Katolikova et al. 2012, Tichy, Pillai et al. 2012).

4.3.2. DNA damage response in ESC

A robust and efficient DNA damage response is of paramount importance for ESC, mostly since any defect in the genome of these cells could be inherited to all cell types of three embryonic lineages. Moreover, given the high proliferative capacity and the unique cell cycle profile of these cells (including G1-S checkpoint deficiency), the accurate and constitutively active DDR and maintenance of genome integrity is even more critical in ESC (Suvorova, Katolikova et al. 2012).

Several studies have reported that, mESC have much lower frequency of undesirable mutations with respect to differentiated cells such as MEF cells, which underlines the fact that ESC get rid of damaged cells either through apoptosis as mESC are hypersensitive to damaging agents, or through highly efficient DNA repair mechanisms. Of note, it has been proved by several groups that mouse ESC predominantly exploit the high fidelity homologous recombination repair (HRR) mechanism to repair their damaged DNA, however differentiated cells mostly use error-prone non homologous end joining (NHEJ) mechanism showing that mESC are inherently different from differentiated somatic cells in terms of their great capacity to repair DNA damage to ensure the genome integrity during early development. Of note, some evidences suggest that treatment with damaging agents may cause premature differentiation of ESC in P53 dependent manner as an alternative way to sustain their genomic stability. (Lin, Chao et al. 2005, Tichy and

Stambrook 2008, Tichy, Pillai et al. 2010, Serrano, Liang et al. 2011, Schneider, Pellegatta et al. 2013, Santos, Faryabi et al. 2014)

It has been demonstrated that the key DNA damage activated kinases (i.e., ATM and ATR) are involved in DDR of ESC. Although ATR and ATM activation is mainly linked to replication stress and DSB, respectively, several evidences proved that ATR activation could also take place upon DSB in ATM dependent manner (Myers and Cortez 2006).

As previously mentioned, ATR knockout is found to be embryonic lethal which could be justified due to very high proliferation rate of ESC which expose them to DNA replication stress (Murga, Bunting et al. 2009). In contrast, ATM knockout mice are born live and could repair DSB in error prone manner (Banuelos, Banath et al. 2008).

4.3.3. High level of γH2AX in mESC is linked to constitutive replication stress

It has been shown previously that both *in vivo* and artificially derived PSC accommodate high amount of γH2AX even in the absence of DNA damaging agents. However, since high level of γH2AX couldn't be linked to DSB formation (due to the absence of co-localization with DSB specific markers such as ATM or 53BP1), the reason for its high phosphorylation was unclear and attributed to specific and undefined PSC chromatin features (Chuykin, Lianguzova et al. 2008, Banath, Banuelos et al. 2009, Ziegler-Birling, Helmrich et al. 2009, Turinetto, Orlando et al. 2012).

Recently, it has been revealed that, phosphorylation of H2AX is directly associated with ATR activation and accumulation of ssDNA during fast replication rounds of ESC, mostly due to their fast G-S transition (Ahuja, Jodkowska et al. 2016). Furthermore, it has been proved that, delaying G1-S transition by Cdc7 inhibitor (which interrupts origin

firing) leads to longer G1 phase in ESC and less amount of γH2AX and replication stress in the following S-phase. This suggests replication-coupled mechanisms that are needed to keep the self-renewal property and genome integrity of ESC (Chuykin, Lianguzova et al. 2008, Ahuja, Jodkowska et al. 2016).

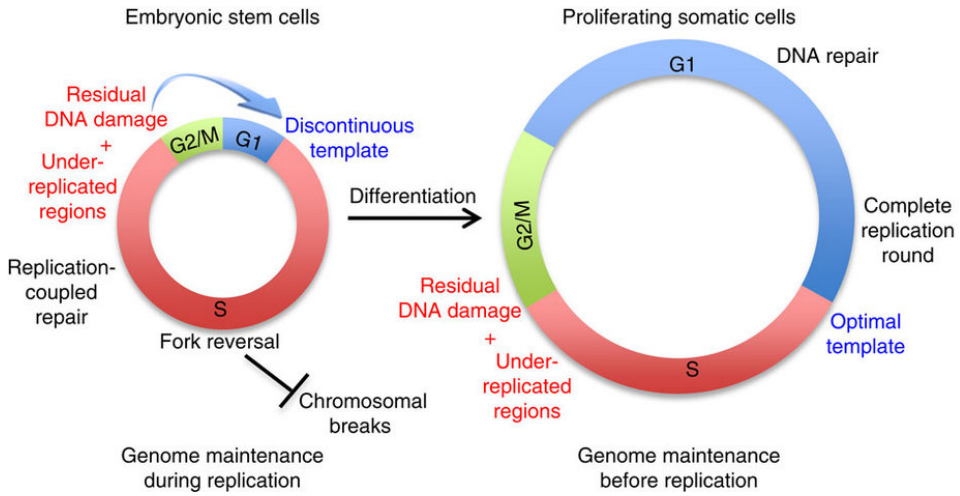


Fig 1.11. Model depicting differential control of genome stability in ESCs and proliferating somatic cells.

Under-replicated regions and residual DNA damage are unavoidably present at the end of each S-phase in both ESCs and somatic cells. However, owing to the brief gap phases, ESCs channel a high number of these lesions in the following S-phase and protect genome integrity by extensive fork reversal and replication-coupled repair. Conversely, differentiated cells have prolonged gap phases, assemble 53BP1 NBs and repair most of these lesions before S-phase entry. Adapted from (Ahuja, Jodkowska et al. 2016).

5. Materials

Reagents and medium components:

High glucose DMEM media - GlutaMAX™-I, Gibco, Life technologies 32430-100

High glucose DMEM, Lonza BE12-614F

RPMI (1640), Lonza BE12-167F

ESC qualified FBS HyClone™ Fetal Bovine Serum (SH30070.03)

Leukemia inhibitory factor (LIF), home made

MEM-Non essential amino acids 100x, Microtech X0557

Fetal Bovine Serum (FBS), North American, Sigma Aldrich F2442

Fetal Bovine Serum, South American, Biowest-S1810-500

Sodium Pyrovate 100mM, Microtech L0642

2-Mercaptoethanol, Life technologies 31350-010

L-glutamine, Microtech X0550

Penicillin Streptomycin, Microtech L0022

Gelatine type A porcine skin, Sigma aldrich G2500-100G

Doxycycline hydrochloride, Sigma Aldrich D9891-5G

KnockOut serum replacement (KSR), Life technologies 10828-028

Leukocyte Alkaline Phosphatase Kit, Sigma Aldrich 86R-1KT

PD 0325901 (Axon medchem, Axon 1408)

CHIR 99021 (Axon medchem, Axon 1386)

RNeasy mini kit (Qiagen, 74104)

SuperScript TM III reverse transcriptase kit (Invitrogen 18080-093)

SsoFastTM EvaGreen® Supermix (Bio-rad)

ATMi (KU-55933), Axon 1367

ATRi (VE 822), Axon 2452

Chk1i (UCN-01)

Cdc7i (PHA767491)

FGF4 (R&D Systems, 235-F4-025)

Heparin (Sigma, H3149)

Puromycin, Vinci-adipogen, AG-CN2-0078-M100

Protease/phosphatase inhibitor cocktail (cell signalling, 5872)

pLVX-EF1α-IRES-Puro vector (Clontech 631988)

pLVX-EF1α-IRES-mCherry cevtor (Clontech 631987)

List of Antibodies:

Alexa Fluor® 647 anti-H2AX- Phosphorylated (Ser139) antibody (Biolegend 613408)

Anti Oct-3/4 antibody (Sanra Cruz, sc-5279)

Anti Nanog (Abcam, ab80892)

Anti SSEA1 (Millipore, MAB4301C3)

Anti 53BP1 (Bethyl laboratories, A300-272)

Anti γH2AX (Millipore, 05-636)

ATR antibody (Cell Signalling, 2790)

Cdc6 antibody (Santa Cruz, sc-9964)

Cdc45 antibody (Santa Cruz, sc-20685)

Phoshpho-Chk1 antibody -S345 (Cell signaling 2348)

Total Chk1 antibody (Santa cruz 8408)

6. Methods

6.1. Cell culture

ESCs were grown in feeder free culture condition either with or without 0.1% gelatin coat and incubated in 37°C and 3% O_2 tension. ESC medium (high glucose DMEM (DMEM Media - GlutaMAX™-I, Gibco)), 15% ESC qualified FBS (HyClone™ Fetal Bovine Serum), 2 mM L-glutamine, 1/500 home-made leukemia inhibitory factor, 0,1 mM non-essential amino acids, 0,1 mM 2-bmercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin, 1mM Sodium Pyruvate) supplemented with two inhibitors (2i from Axon medchem) that are PD 0325901; highly specific non-ATP-competitive inhibitor of MEK (aka MKK) 1/2 at final concentration of 1uM and CHIR 99021; specific glycogen synthase kinase GSK-3 inhibitor at final concentration. MEF cells were cultured in MEF medium (high glucose DMEM (Lonza), 10% north America FBS, 2 mM L-glutamine, 0,1 mM non-essential amino acids, 50 units/ml penicillin, 1mM Sodium Pyruvate and 50 mg/ml streptomycin).

6.2. Treatments

All treatments including APH, HU, UV, ATRi, ATMi, Cdc7i, Chk1i were added in to fresh ESC medium and the cells were kept in 37°C and 3% O_2 tension for overnight (16-17 hrs) unless mentioned in each particular result section. APH was used at various concentrations ranging from 0.3uM to 6uM that is mentioned in each experiment in order to induce mild or acute replication stress response. Similarly, HU was used at 0.1mM-3mM. ATMi (KU-55933) and ATRi (VE 822) were used at the concentration of 10uM and 1uM respectively. Moreover, Chk1i (UCN-01) at the concentration of 100nM and Cdc7i

(PHA767491) at the concentration of 5uM were used. UV radiation was performed at the dosage ranging from 2 to10 J/m^2.

6.3. Flow cytometry (FACS)

Zscan4-Emerald ESCs were fixed and permeabilized using Cytoperm/Cytofix kit (BD Biosciences), and subsequently stained for one hour at room temperature with Alexa Fluor® 647 anti-H2AX- Phosphorylated (Ser139) antibody (Biolegend). Cells were washed and acquired on a FACS Calibur instrument. For Caspase3 FACS analysis after fixation and permeabilization, Caspase3 antibody were used for one hr at RT followed by washes and one hr incubation in donkey anti rabbit secondary antibody. For Zscan4-Emerald-GFP acquisition cells were trypsinized, collected and subsequently acquired without fixation step.

6.4. RNA extraction, cDNA synthesis and qPCR

RNA was extracted using RNeasy mini kit (QIAGEN), quantified by NanoDrop spectrophotometer and cDNA was prepared from 2 μg total DNA-depleted RNA using SuperScript TM III reverse transcriptase kit (Invitrogen cat# 18080-093) following manufacturer's instructions. qPCR assay performed based on standard protocol using 10X SsoFastTM EvaGreen® Supermix, 10 μM primer mix and a total amount of cDNA corresponding to 5 ng of starting RNA for each reaction. For the list of oligos used in this study please refer to (Takahashi and Yamanaka 2006, Macfarlan, Gifford et al. 2012).

6.5. Immunocytochemistry

Briefly cells fixed in 4% PFA and subsequently blocked for one hour in FBS (10%) and Triton (0.1%). Then, cells were incubated with primary antibody at 4°C overnight, followed by washes and incubation with secondary antibody for an hour at room temperature. Next samples were mounted and images were acquired with wide field florescent microscope. Antibodies used in this study were Anti Oct-3/4 (Sanra Cruz sc-5279), Anti Nanog (Abcam ab80892), Anti SSEA1 (Millipore MAB4301C3), Anti 53BP1 (Bethyl laboratories #A300-272), Anti γH2AX (Millipore 05-636). Acquired figures were analyzed with ImageJ software.

6.6. Immunoblotting

The cells were trypsinized and washed with PBS and then lysed in RIPA buffer plus protease/phosphatase inhibitor cocktail (cell signalling, #5872), incubating for 30 minutes on a rotating wheel at +4°C. Lysates were sonicated with a Bioruptor Sonication System (UCD200) at high power for 3 cycles of 30 seconds with one minute breaks. Lysates were centrifuged at 13000 rpm for 20-30 minutes and clear supernatants were transferred to new tubes. The proteins were quantified using Bio-Rad protein assay and following manufacturer's instructions. For the detection of each protein, 35 µg of total protein extracts were loaded. Standard western blot was performed using following antibodies: ATR antibody (Cell Signalling 2790), Phoshpho-Chk1 antibody -S345 (Cell signaling 2348) Cdc6 antibody (Santa Cruz sc-9964), and Cdc45 antibody (Santa Cruz sc-20685).

6.7. Target preparation for microarray

The quality of total RNA was first assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Biotin-labeled cDNA targets were synthesized

starting from 150 ng of total RNA. Double stranded cDNA synthesis and related cRNA was performed with GeneChip® WT Plus Kit (Affymetrix, Santa Clara, CA). With the same kit was synthesized the sense strand cDNA before to be fragmented and labeled. All steps of the labeling protocol were performed as suggested by Affymetrix. Each eukaryotic GeneChip® probe array contains probe sets for several B. subtilis genes that are absent in the samples analyzed (lys, phe, thr, and dap). This Poly-A RNA Control Kit contains in vitro synthesized, polyadenylated transcripts for these B. subtilis genes that are pre-mixed at staggered concentrations to allow GeneChip® probe array users to assess the overall success of the assay. Poly-A RNA Controls final concentration in each target are lys 1:100,000, phe 1:50,000, thr 1:25,000 and dap 1:6,667.

6.8. DNA Strip hybridization

Hybridization was performed using the GeneAtlas™ Hybridization, Wash and Stain Kit. It contains a mix for target dilution, DMSO at a final concentration of 10% and pre-mixed biotin-labeled control oligo B2 and bioB, bioC, bioD and Cre controls (Affymetrix cat# 900299) at a final concentration of 50 pM, 1.5 pM, 5 pM, 25 pM and 100 pM respectively. Targets were diluted in hybridization buffer at a concentration of 0.05 µg/ul, denatured at 99 °C for 5 minutes, incubated at 45 °C for 5 minutes and centrifuged at 5,000 rpm for 1 minute. The Array Strip is then moved to a hybridization tray, which contains the hybridization cocktails. A single Gene 2.1 PEG Array Strip is hybridized with four different biotin-labeled targets. Hybridizations were performed for 20 hours at 48°C in the GeneAtlas™ Hybridization Station. The Array Strips were washed and stained in the GeneAtlas™ Personal Fluidics Station. At the end of this procedure the array strips were placed into the imaging tray, which contains the Array Holding buffer.

6.9. Image acquisition, processing and bioinformatics analysis

The array strips were imaged using the GeneAtlas™ Imaging Station. Affymetrix GeneAtlas™ Command Console software was used to acquire the Array Strip images and generate.

Differential expression analysis

We imported the microarray CEL files into R-3.2.2 (R Core Team. 2015. R: A Language and Environment for Statistical Computing. https://www.r-project.org/ (Accessed September 12, 2016) and normalized intensity values with RMA (Irizarry, Hobbs et al. 2003) normalization. We performed differential expression analysis using the limma (Ritchie, Phipson et al. 2015) package, weighting arrays with the arrayWeights function (Ritchie, Diyagama et al. 2006) and calculating p-values with an empirical Bayes method (Smyth 2004).

Expression Z-scores were calculated using the probe set level RMA normalized microarray intensity values using the standard formula:

$$Z - score = \frac{x - \mu}{\sigma}$$

Where x is the RMA normalized microarray intensity, μ is the probeset level average intensity value across all samples, and σ is the probeset level standard deviation of the intensity values.

Gene set enrichment

The CAMERA gene set enrichment p-values were calculated using the limma package and the camera function (Wu and Smyth 2012). Gene sets were defined based on the MsigDB gene sets (Subramanian, Tamayo et al. 2005), using a mouse orthologous collection (Hu Y. 2016. Mouse and human orthologues of the MSigDB in R format. http://bioinf.wehi.edu.au/software/MSigDB/ (Accessed September 12, 2016).

In the GSVA based enrichment analysis, we used the MsigDB gene sets, and transformed the individual gene expression values into gene set expression values with the GSVA package (Hanzelmann, Castelo et al. 2013). We calculated the gene set differential expression as in the gene level differential expression analysis, only skipping the RMA normalization step.

6.10. Genotyping of ATR Seckel ESC

In order to obtain homozygous ATR Seckel ESC, all nine ESC lines derived from breeding of ATR Seckel heterozygous mice were genotyped using previously described primer sets (Murga, Bunting et al. 2009). 300bp and 545 bp bands correspond to WT and ATR Seckel -/- genotypes.

6.11. *In-vitro* differentiation toward giant trophoblast-like cells

ATR Seckel and WT ESCs were seeded on gelatin coated 6-well plate in ESC medium and 24 hours post treatment, medium was changed to trophectoderm stem cell (TSC) differentiation medium, which contains: 30% RPMI 1640 (with 20% FBS, 1mM pyruvate, 2mM L-glutamine, 100 mM β-mercaptoethanol), 70% of conditioned medium from mitomycin-C-inactivated fibroblasts, 25 ng/ml of FGF4 (R&D Systems, 235-F4-025) and 1 ug/ml of heparin (Sigma, H3149). We changed the medium with fresh one every other day to maintain TSCs. To induce giant cell differentiation established TSC were split at day 2 on gelatin coated plate. After 24 hrs medium was changed to RPMI 1640 (with 20% FBS, 1mMpyruvate, 2mML-glutamine, 100 mM β-mercaptoethanol) in the absence of heparin and FGF4, therefore, we changed the medium every other day for 3 days.

6.12. Mouse chimaera assay

Briefly, morula stage embryos were bought from Charles River laboratories. They were thawed few hours prior to injection and about five to eight ESC were injected into the perivitelline space of high quality morula-stage embryos using laser-assisted technique and glass micro-capillary tubes. Finally, embryos were cultured for 24 hours at 37C, 5% CO2 to allow the blastocysts development prior to the microscopic analysis.

6.13. Molecular Cloning

The sequences corresponding to mouse Cdc45 and Cdc6 cDNA were amplified by polymerase chain reaction from Origene Clones (NM_009862 and NM_001025779) using the primers below and cloned into the pLVX-EF1α-IRES-Puro (#631988) vector from Clontech and were transformed in TOP10 Chemically Competent E. coli (Invitrogen) and sequenced for checking the accuracy of the clone sequences.

1) Mouse-Cdc45-SpeI-Fw: 5'-GACTAGTACCATGTTCGTGACCGATTTCCGCAAG-3'

2) Mouse-Cdc45-FLAG-BamHI-R:

5'-

CGCGGATCCTCACTTGTCATCGTCGTCCTTGTAGTCTCCTCCTCCGGACAGCAG

TGACACAAGAG-3'

3) Mouse-Cdc6-SpeI-Fw: 5'-GACTAGTACCATGCCTCAAACCAGATCCCAGACA-3'

4) Mouse-Cdc6-HA-BamHI-R:

5'-

CGCGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTATCCTCCTCCGGGCA

GACCAGCAGCG-3'

6.14. Lentivirus production

pLVX-EF1α-IRES-mCherry vector was co-transfected with plasmids that are expressing POL, REV, TAT, and the vesicular stomatitis virus envelope glycoprotein (VSV-G) into 80% confluent 293T cells using calcium phosphate precipitation in the presence of 25mM chloroquine. The supernatant of transfected cells were collected every 24 hrs for two days and concentrated using PEG-itTM virus precipitation solution and viral particles were re-suspended in DMEM and frozen in small aliquots in -80.

6.15. Somatic cell reprogramming

Somatic cell reprogramming was carried out using Dox-inducible polycistronic lentiviral vector STEMCCA, which consists all four reprogramming factors Oct4, c-Myc, Klf4 and Sox2 (Sommer, Stadtfeld et al. 2009). 10^5 low passage Oct4-GFP MEFs were seeded at clonal density in 6-well plate on day0. On the next day, cells were infected with STEMCCA and rtTA viruses in the presence of 8ug/ml polybrene. On day2, infected cells were trypsinized, counted and re-plated on mitomysin-inactivated MEF feeder layer. The day after MEF medium was replaced with iPSC medium (high glucose DMEM, 15% knockout serum replacement-Invitrogen-, 2 mM L-glutamine, 1/500 home-made leukemia inhibitory factor, 0,1 mM non-essential amino acids, 0,1 mM 2-bmercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin) supplemented with 1 ug/ml doxycycline to induce expression of the reprogramming factors and medium was changed daily till colonies started to emerge. Around day 6 small morphological changes started to appear and iPSC colonies emerged after about two weeks of reprogramming induction. On day 20, Dox was withdrawn from medium and a week later each *bona fide* iPSC were picked with needle and expanded on a MEF feeder layer to establish individual clones for further characterization.

6.16. Alkaline phosphatase assay

Alkaline phosphatase (ALP) is a hydrolase enzyme, which removes phosphate groups from various molecules, such as alkaloids and proteins. The undifferentiated and self-renewing ICM-derived mESC, have a high ALP activity (Johnson, Calarco et al. 1977). To test the pluripotency features of iPSC I performed ALP assay using Alkaline Phosphatase Detection Kit (Sigma) and acquired the images using an Olympus AX70 wide field microscope.

6.17. In-vitro differentiation of PSC toward three embryonic germ layers

To induce embryoid body formation, four million iPSCs were plated in non-adherent bacterial dish in 15 ml EB medium (iPSC medium without LIF plus 10% FBS) for four days and the medium was replaced with fresh medium every other day. At day five, EBs were re-plated on gelatin-coated adherent dishes for further differentiation and the medium was changed every other day for another week.

6.18. Definition of the mathematical model and solution

The model describes the dynamics of the number of positive and negative cells. Positive cells stochastically duplicate with rate b+, die with rate d+, and switch to negative state with rate k+; negative cells duplicate with rate b-, die with rate d-, and switch to positive state with rate k-. Mean-field deterministic rate equations for these processes can be obtained:

$$\frac{\mathrm{d}N_+}{\mathrm{d}t} = q_+ N_+ - k_+ N_+ + k_- N_-$$

$$\frac{\mathrm{d}N_-}{\mathrm{d}t} = q_- N_- + k_+ N_+ - k_- N_-$$

(1)

Here, N+ and N- are the numbers of positive and negative cells respectively, and birth and death rates appear only through the combinations

$$q_+ := b_+ - d_+$$
$$q_- := b_- - d_-$$

Eq (1) can be solved analytically. The general solution is a complicated expression, which we do not quote here. To give an example, by taking the initial condition

$$N_+(0) = 0$$
$$N_-(0) = N_0$$

one gets the following expression for the dynamics of the number of Zscan4 positive cells:

$$N_+(t) = \frac{2N_0 k_- \mathrm{e}^{\frac{1}{2}t(-k_- - k_+ + q_- + q_+)} \sinh\left(\frac{1}{2}t\sqrt{k_-^2 + 2k_-(k_+ - q_- + q_+) + (k_+ + q_- - q_+)^2}\right)}{\sqrt{k_-^2 + 2k_-(k_+ - q_- + q_+) + (k_+ + q_- - q_+)^2}}$$

Not all combinations of parameters give rise to a mixed long-time state, with non-zero fraction of positive and negative cells. In fact, a condition for this to happen is $q_+ = q_-$, i.e., the equality of the total net growth rates (duplication minus death) for the two cell states.

In this case, the rate equations in Eq. (1) imply a simple closed system of equations for the fractions of positive and negative cells, defined as

$$\phi_+ := N_+/(N_+ + N_-)$$
$$\phi_- := N_-/(N_+ + N_-)$$

Specifically,

$$\frac{\mathrm{d}\phi_+}{\mathrm{d}t} = -k_+\phi_+ + k_-\phi_-$$

$$\frac{\mathrm{d}\phi_-}{\mathrm{d}t} = +k_+\phi_+ - k_-\phi_-$$

(2)

Whenever $q_+ = q_-$, Eq. (2) shows that the stable fraction of positive cells is

$$\phi_+^{(\infty)} = \frac{k_-}{k_+ + k_-}$$

(3)

Comparison of Model and Data

Full solution of Eq (1) shows that, in general, if the net growth rates q_+ and q_- are unbalanced, such long-time stable fraction of treated *vs* untreated cells is not reached. Hence, we suppose that this balance condition is fulfilled for the control experiment, for which a stationary mixed population is reached after approximately 4 days. Under this condition, Eq. (3) can be used to estimate the combination of model parameters appearing in the right-hand side from the long-time values. We estimated errors bars from a standard multi-branch fit of the solution to Eq. (2) against data with the two different initial conditions (100% and 0% Zscan4-positive cells). This fit gives:

$$k_+ = 0.16 \pm 0.03 \ \mathrm{d}^{-1}$$
$$k_- = 0.84 \pm 0.08 \ \mathrm{d}^{-1}$$

We tested; by using the model, whether the experimental data in treated conditions may be reproduced in the null scenario where the switching rates k_+ and k_- are the same as in the control. Importantly, the model applies to conditions where the parameters, and in particular the switching rates are constant. If the experiment with drug treatment is prolonged for multiple days, the drug is diluted (in a way that is not completely controlled). Therefore, it makes sense to compare data and model after one day of treatment. Any measurement at later times would need to fix unknown time-varying parameters in the model, which is unfeasible with the current resolution and statistics of the data.

Since the drug treatment can affect division and death rates differently, q_+ and q_- can be (and are likely) different in this case. Hence, when comparing the model to the result in the treated condition one cannot use the assumption that a stable state is reached by the system at long times, other than the trivial one with 100% positive cells. However, with fixed k_+ and k_-, a relation between q_+ and q_- must be satisfied in order to reproduce the empirical cell counts in the treated cultures from day 0 to day 1. This constraint is imposed using the full analytical solution to Eq. (1).

This operation constrains the model up to only one free parameter (q_+). Plotting the possible theoretical curves for the time course of the fraction of Zscan4-positive cells by varying q_+ (Fig. 1) shows that the experimental data at day 1 can be reproduced only if q_+ is at least a factor f=2.2 larger than it is in the control (considering the effect of the uncertainty in the parameters k_+ and k_-, the fold change factor f lays in the range 1.8 – 2.7). This would correspond to an extremely unrealistic situation in terms of changes in division and death rates determined by the drugs, which could be observable in only one day. For example, if the death rate is negligible in the untreated conditions, then, even taking the worst case scenario that in the treated conditions the death rate is unaffected, treated cells would have to divide 2-3 times per day, which is unrealistic. Hence, this analysis supports the conclusion that changes in the switching rates are determined by the effect of the drug.

7. Results

Although the extended developmental potential of 2C-like cells in generating embryonic plus extra-embryonic tissues as well as their higher reprogrammability through SCNT assay has been explored by several groups (Macfarlan, Gifford et al. 2012, Amano, Hirata et al. 2013, Ishiuchi, Enriquez-Gasca et al. 2015), the signal triggering reactivation of totipotency genes in PSC culture and the exact molecular mechanism underlying the reactivation of 2C-like transcriptional signature is not well investigated yet (Fig 7.1).

Due to the critical role of Zscan4 and some other 2C-like genes expression in regulating the genomic stability of PSC and previously reported constitutively active replication stress response in mouse embryonic stem cells under normal culture condition (Ahuja, Jodkowska et al. 2016), here we asked if the expression of Zscan4 and activation of 2C-like genes network are controlled by activation of the DNA damage response pathways as a unique characteristic feature of PSC.

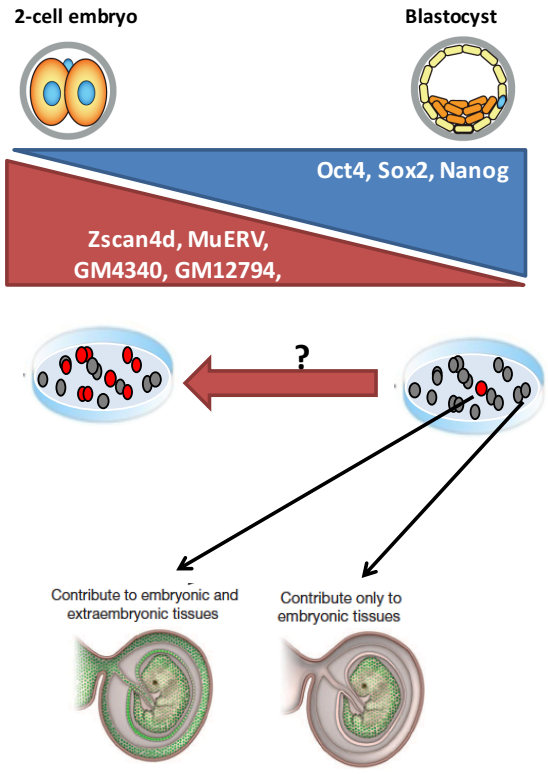


Fig 7.1. The molecular mechanism underlying transition to totipotent-like state is unknown.

About 1-5% of cells in PSC culture express 2C-like specific genes such as Zscan4, MuERV-L, Gm4340 and Gm12794. 2C-like cells have shown higher potency in functional assay, however, the exact molecular mechanism that regulates transitioning to such state is not yet known.

7.1. ATR mediated DNA damage response triggers activation of key 2C-like specific genes in mESC

It has been shown that transient burst of Zscan4 expression, play a critical role in maintaining the genomic stability of ESC (Zalzman, Falco et al. 2010). Moreover, several studies have demonstrated that the DNA damage response (DDR) is constitutively active in ESC due to high proliferation rate and constitutive replication stress, (Chuykin, Lianguzova et al. 2008, Banath, Banuelos et al. 2009, Ziegler-Birling, Helmrich et al. 2009, Ahuja, Jodkowska et al. 2016). Therefore, we tested whether Zscan4 expression level is associated with constitutively active replication stress response in ESC under normal culture condition. To test this hypothesis, we first examined the basal level of γH2AX as an indicator of DDR activation in Zscan4-Emerald ESC line. Interestingly, flow cytometry analysis revealed a significant enrichment of γH2AX positive cells within Zscan4-Emerald positive population with respect to Zscan4-Emerald negative ESC population (Fig 7.2A).

Hence, we asked if the expression of the key totipotency specific genes such as Zscan4 and MuERV-L is responsive to the further activation of replication stress response through extrinsic stimuli. To this end, we exposed Zscan4-emerald ESC to aphidicolin (APH) and hydroxyurea (HU), two well-known agents that induce replication stress and subjected them to FACS analysis. As shown in Fig.7.2B and C, in APH and HU treated conditions, the proportion of Zscan4 positive cells was increased significantly in comparison to untreated control (CNT).

Next, to dissect the contribution of the main apical kinases in reactivation of 2C-like specific genes in PSC culture, we asked if suppression of DDR pathways could restrain the activation of main totipotency genes under normal and DNA damage response

inducing condition. Thus, we inhibited DDR by addition of specific ATM and ATR inhibitors (KU-55933 and VE 822, respectively) in Zscan4-Emerald ES cells. Flow cytometry analysis revealed that the addition of ATR inhibitor could remarkably reduce the number of Zscan4-Emerald positive cells both under normal culture condition but also upon APH and HU treated conditions (Fig. 7.2B-C). However, ATM inhibitor did not exert a significant effect on the reversion of totipotency specific genes; neither in normal nor under APH and HU treated circumstances.

Strikingly, in line with the recent report where constitutive replication stress in embryonic stem cells is associated to their short G1 phase (Ahuja, Jodkowska et al. 2016), we found that delaying G1 phase by addition of CDC7 inhibitor could significantly restrain the Zscan4 positive cells in ESC culture. In addition, similar to ATR inhibition, treatment of ESC with CHK1 inhibitor reduced the percentage of Zscan4 positive cells in untreated but also APH treated condition (Fig 7.2D).

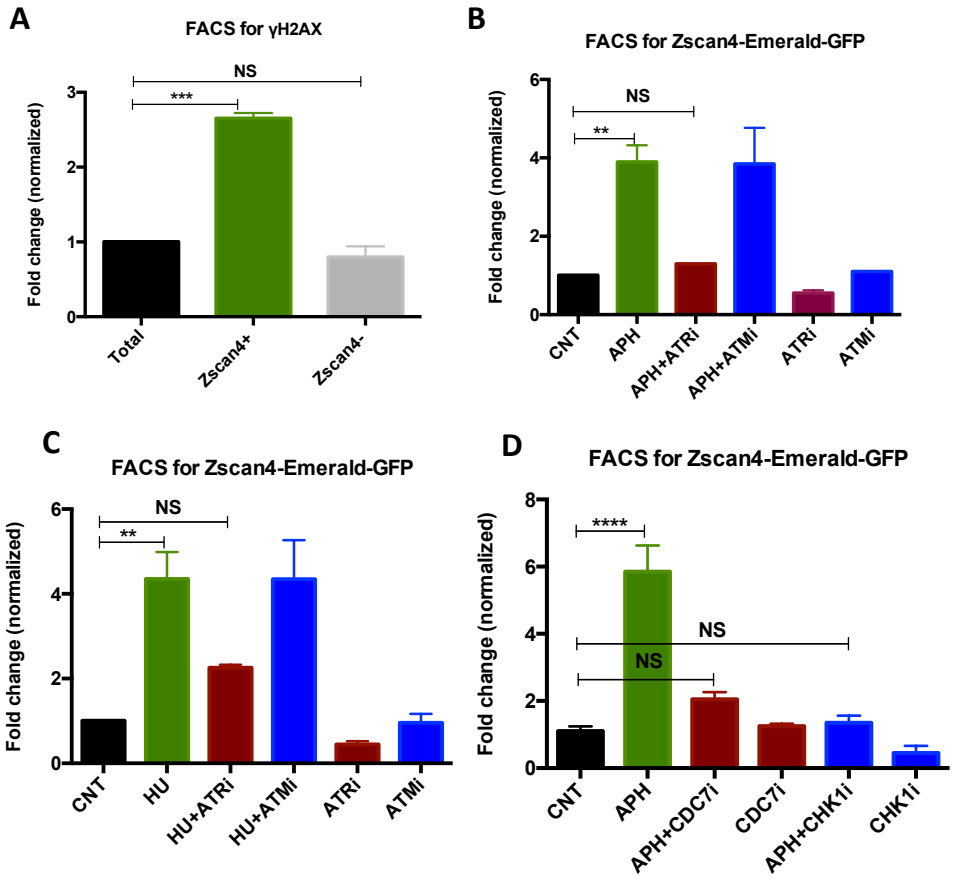


Fig 7.2. ATR mediated DNA replication stress triggered activation of "Zscan4", a key 2C-like specific gene in mESC

A) FACS analysis on sorted Zscan4-Emerald-ESC for DNA damage marker γH2AX. **B,C)** FACS analysis revealed that Zscan4 positive population increased in ESC culture upon replication stress induction with APH and HU. ATRi but not ATMi reduced the percentage of Zscan4+ population. **D)** FACS analysis on Zscan4-Emerald ESC indicated that Chk1 inhibition and G1 phase prolongation (Cdc7i) decreased the percentage of Zscan4+ population.

Of note, the number of Zscan4 positive population both under normal condition and upon APH treatment, clearly correlated with the number of γH2AX positive cells (Fig. 7.3) as in normal culture condition higher proportion of cells were negative for both γH2AX and Zscan4 while upon APH treatment, γH2AX and Zscan4 positive populations were significantly increased. Importantly, ATR inhibition (but not ATM) dropped the percentage of γH2AX and Zscan4 positive populations back to the control level. (Fig. 7.3).

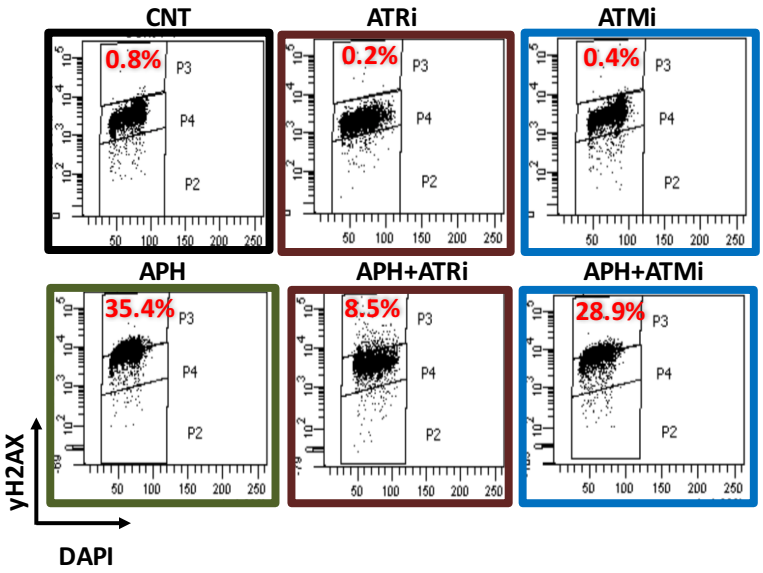


Fig 7.3. FACS analysis on Zscan4- Emerald ESC showing the percentage of γH2AX+ population, upon treatment with APH and/or ATRi or ATMi.

Next, we exposed ESC to the increasing dosage of aphidicolin (APH), hydroxyurea (HU) and ultraviolet light (UV) which could activate DNA damage response pathways. Strikingly, qPCR and FACS analysis showed that the expression of Zscan4 and MuERV-L were significantly upregulated upon all treatments in a time and dosage-dependent manner in several independent ESC lines (Zscan4- emerald, E14 and R1) (Fig 7.4A-F). Finally, we found that APH treatment do not alter the expression of MuERV-L in mouse embryonic fibroblast (MEF) lines with distinct genetic backgrounds (C57BL/6 and 129 strains). Moreover, Zscan4d expression was not detectable using qPCR in these MEF lines, suggesting that the activation of totipotency related genes could be cell type specific (Fig 7.4 G).

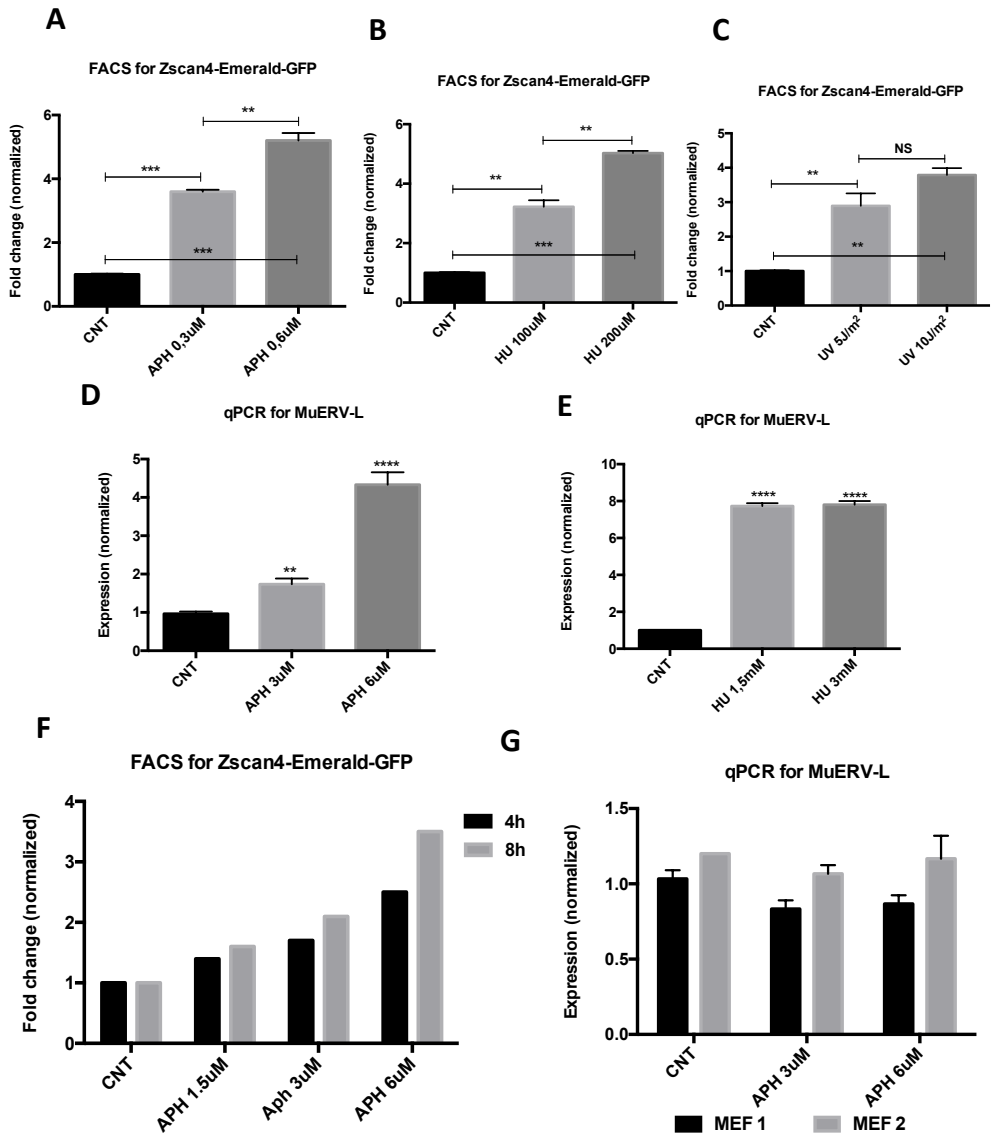


Fig 7.4. Wide range of replication stress inducing agents in PSC trigger activation of key totipotency genes in dosage dependent manner.

A,B,C) FACS analysis on Zscan4-Emerald ESC for Zscan4-GFP-Emerald expression upon treatment with various concentrations of APH, HU and UV. **D,E)** qPCR for MuERV-L, one of the main 2C-sepecifc gene on E14 lines treated with APH or HU. **F)** FACS analysis on Zscan4 ESC showed the time and concentration dependent increase in Zscan4 positive population. **G)** qPCR results showed that expression of MuERV-L is not altered upon APH treatment in MEF cells even at higher concentrations. (Zscan4 is not detected by qPCR).

These results overall suggest that emergence of Zscan4 positive cells in ESC culture is regulated through ATR-mediated DNA damage response (Fig 7.2-7.4).

7.2. Aphidicolin treatment reactivated the transcriptional signature of 2C-like specific genes, through ATR dependent pathway.

Next, to understand if activation of DNA damage response could result in global transcriptional alterations similar to that of 2 cell-stage embryos, we performed global gene expression profiling on E14 and R1 cells (two established ESC lines) upon aphidicolin treatment. Analysis of differentially expressed genes (DEGs) (False discovery rate (FDR)-adjusted P-value<0.05) identified 74 upregulated genes with more than two-fold changes in gene expression upon APH treatment, and only 9 downregulated genes, which is in agreement with general openness of the chromatin in totipotent state (Ishiuchi, Enriquez-Gasca et al. 2015) (Fig 7.5).

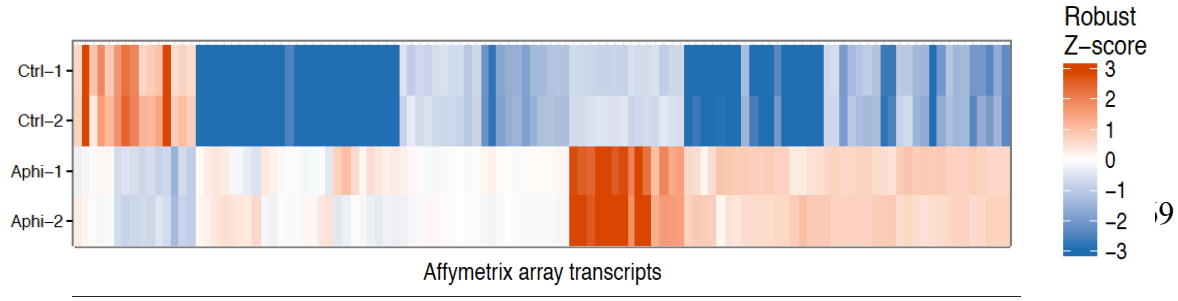


Fig 7.5. Microarray heatmap analysis showed upregulation of genes rather than downregulation upon APH treatment, in line with general openness of chromatin in totipotent-like state.

To understand how many of the identified differentially expressed genes (DEGs) overlap with those specifically expressed in 2C embryos or 2C-like cells, we compared our DEGs (APH treated condition v.s. CNT) with those of previously published datasets (Macfarlan, Gifford et al. 2012). Through such comparison, we found that 46 out of 83 (55%) DEGs overlap with 2C-specific genes that are previously identified by Macfarlan, et al in 2012 (Fig. 7.6).

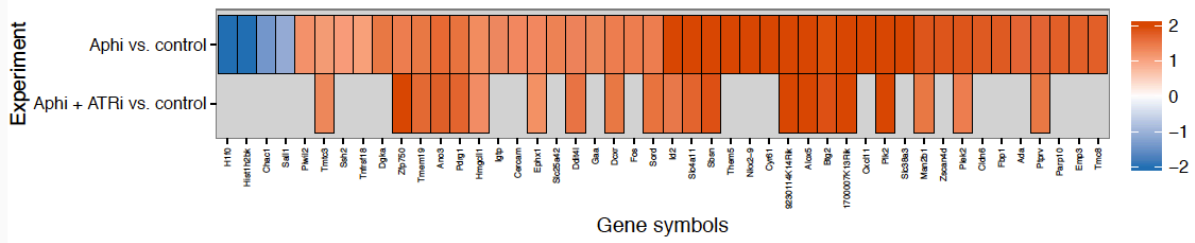


Fig 7.6. APH treatment reactivated transcriptional signature of 2C-like specific genes, through ATR dependent pathways.

Comprehensive heatmap analysis indicated that APH treated ESC have a strong enrichment for 2C-specific genes and the large portion of these totipotency related genes were rescued through ATR inhibition.

Next, I validated the most important 2C-like related genes found to be upregulated in previous works (Zalzman, Falco et al. 2010, Macfarlan, Gifford et al. 2012, Ishiuchi, Enriquez-Gasca et al. 2015) including GM4340, Gm12794, Zscan4 family genes (Zscan4c, Zscan4d and Zscan4-Total), Tcstv3, Tdpoz3 and Eif1a through qPCR assay (Fig 7.7 A, B).

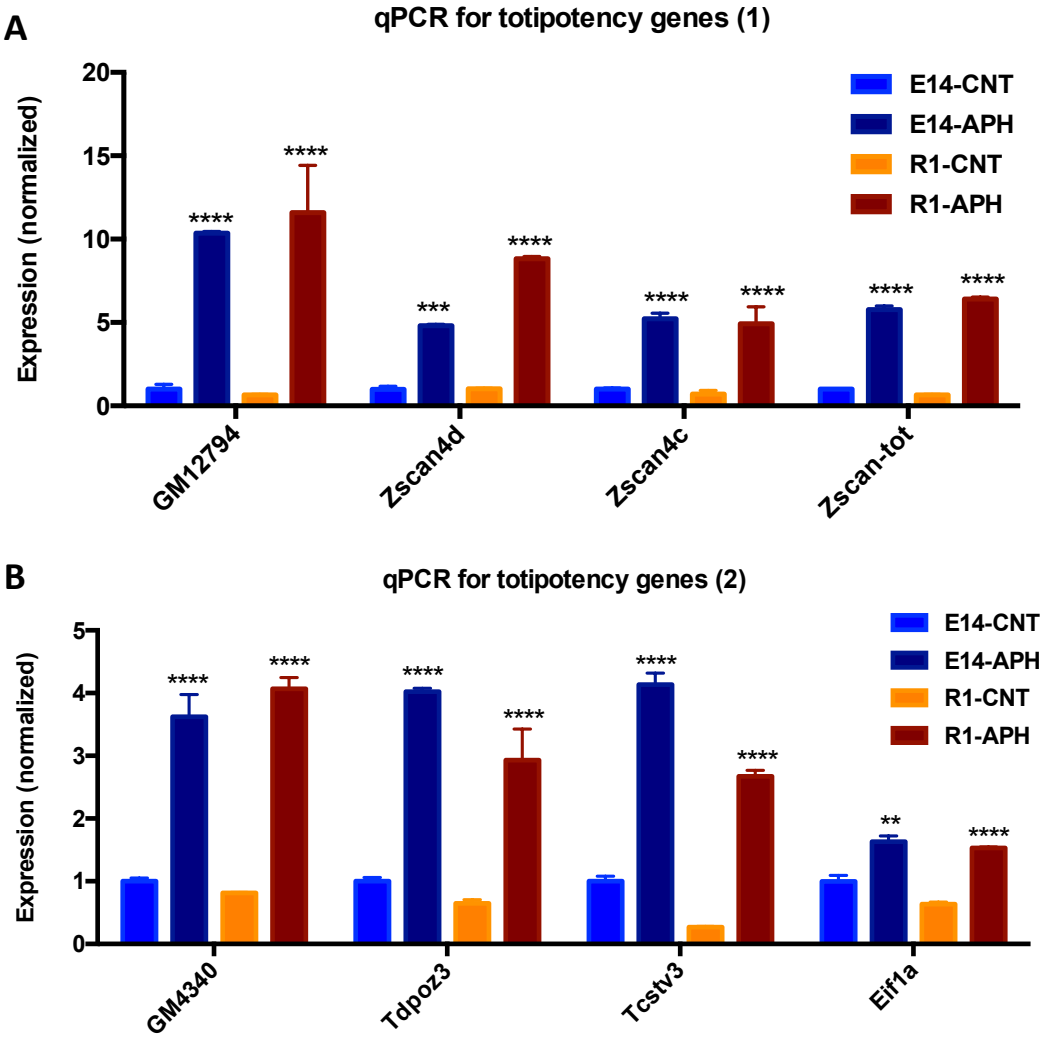


Fig 7.7. Validation of Microarray results by performing qPCR.

A,B) qPCR results showed over expression of several key totipotency genes in APH treated ESCs.

Two cell (2C)-like cells have been shown to reduce the expression of the pluripotency markers at the protein but not transcriptional level (Macfarlan, Gifford et al. 2012, Ishiuchi, Enriquez-Gasca et al. 2015). Here, I confirmed that Zscan4 expressing ESC are negatively stained for Oct4 and Nanog pluripotency markers (Fig 7.8E). Thus, in order to understand if DNA damage induced cells share such features with the 2C-like cells, we

checked the expression of the canonical pluripotency markers upon APH treatment. In agreement with previous publications, we found a mild down regulation of Nanog, Pou5f1 (Oct4) upon APH treatment by immunostaining (Fig 7.8B). However, no significant alterations in the expression of pluripotency related (Sox2, Nanog and Pou5f1) or germ layer specific (Gata6, Wt1 or Pax6) genes were observed at the transcriptional level suggesting that these cells are not biased to differentiate toward germ layer-specific lineages under replication stress (Fig 7.8C-D). Of note, replication stress induced totipotent-like cells (RSIT-LC), gained flat-like morphology, which was distinct from very compact and multilayer culture of untreated mESC CNT (Fig 7.8A) consistent with previous findings (Amano, Hirata et al. 2013, Ko 2016).

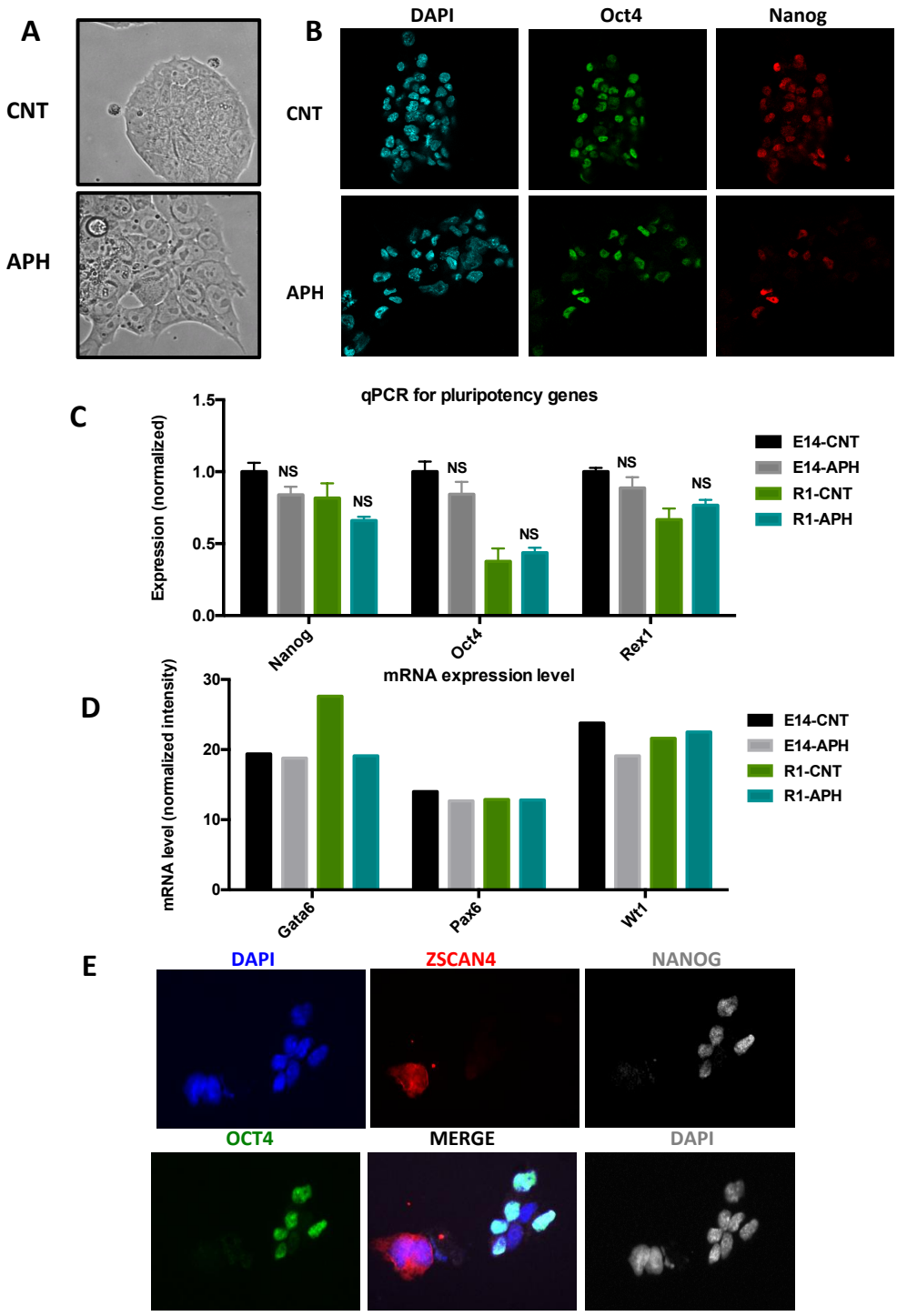


Fig 7.8. Characterization of replication stress induced 2C-like cells.

A) Wide field images showing morphological changes in APH treated ESC versus untreated control. **B)** Immunostaining for pluripotency markers on Zscan4-emerald ESC showed that the expression of Nanog and Oct4 is mildly decreased upon APH treatment. **C)** qPCR results in E14 and R1 ESC lines, revealed that there was no significant alteration

of pluripotency genes at mRNA level. **D)** mRNA level in both E14 and R1 ESC lines showed that the expression of three germ line specific markers upon aphidicolin treatment was comparable to untreated control. **E)** Immunostaining results illustrated that Zscan4-positive 2C-like cells do not express Nanog and Oct4 at protein level (NS=not significant, each condition was compared to the corresponding control).

Next, we aimed to nail down the contribution of ATR on the global transcriptional reversion of replication stress induced 2C-spesific genes. Of note, the geneset enrichment analysis confirmed a robust silencing of DNA repair signaling pathway upon addition of specific ATR inhibitor (Fig 7.9A). Moreover, we also found activation of UV response related gene categories, which could further indicate the activation of replication stress upon APH treatment. Strikingly, the heatmap analysis identified considerable portion 54% (25 out of 46) of genes which has reverted their expression back to the level of control condition, upon addition of ATR inhibitor (Fig 7.6). In addition, qPCR results for MuERV-L and Zscan4d genes validated the effect of ATR inhibition on restraining the expression of 2C- specific genes, upon treatment with either APH or HU (Fig 7.9B-E).

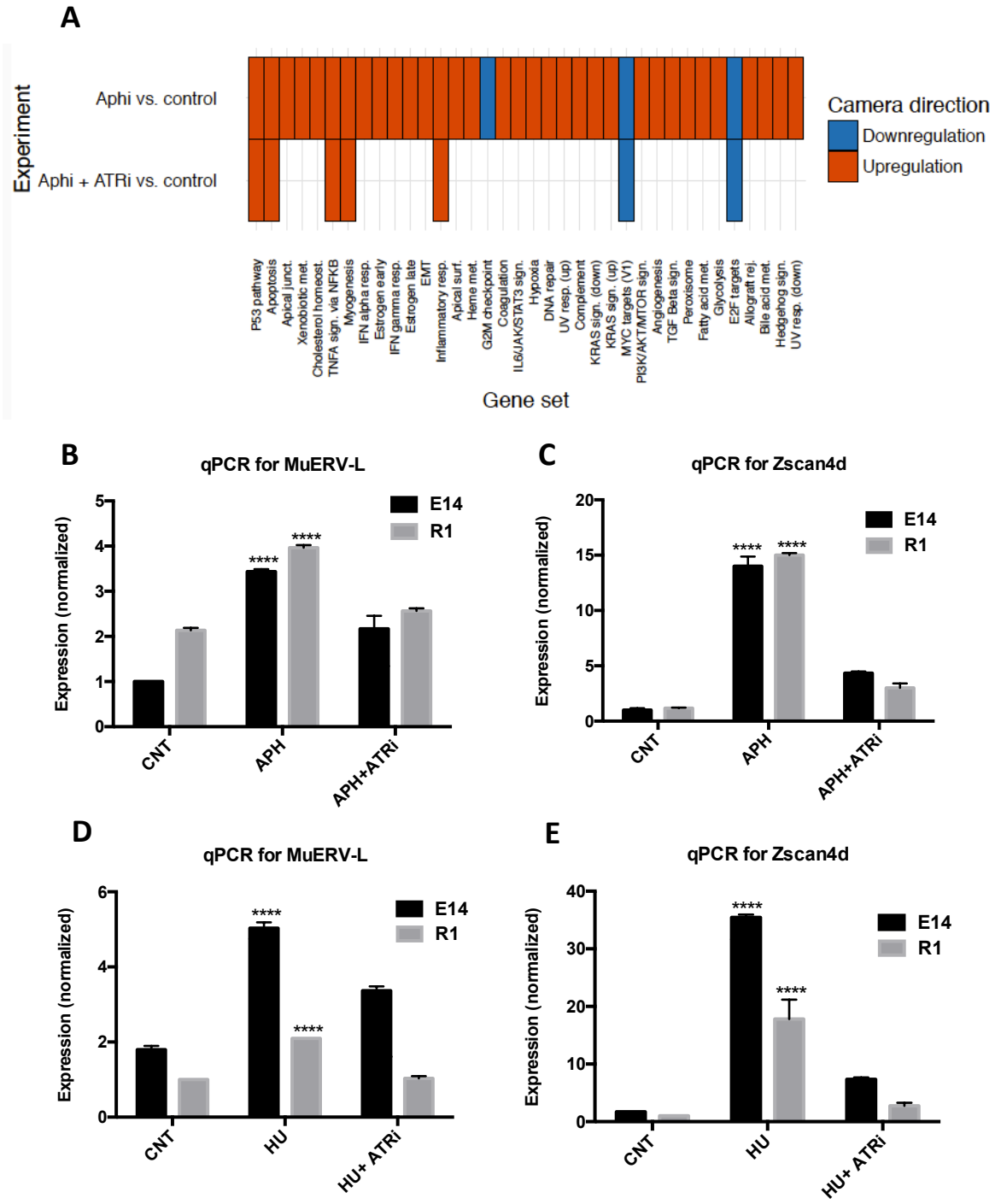


Fig 7.9. Expression of key totipotency genes are regulated through ATR dependent replication stress response

A) Camera heatmap analysis. **B-E)** qPCR data representing that ATR inhibitor rescued the expression of Zscan4d and MuERV-L, two key 2C specific genes upon APH and HU treatment in E14 and R1 ESC.

7.3. ATR Seckel ESC have significantly lower expression level of totipotency genes

Next, in order to confirm that ATR inhibition negatively regulates the reactivation of 2C-like genes and to exclude any unspecific inhibitory effect of ATRi that is used in this study, we aimed to generate ATR deficient ESC and since complete ablation of ATR is embryonic lethal we aimed to establish ATR Seckel ESCs from ATR Seckel mice that have been originally generated by Capetillo group. To this aim, two heterozygous mouse for ATR Seckel allele were crossed and ESC were isolated at embryonic day 3.5 (E3.5). Genotyping results showed that among nine isolated ESC lines, two were homozygous for ATR Seckel that were used for further analysis (Fig 7.10A). Of note, we did not observe any significant differences in the expression of key pluripotency genes in ATR Seckel ESC with respect to wild type controls (Fig 7.10 B). Expectedly, immunoblot results showed that the level of ATR protein in ATR Seckel ESCs is markedly decreased in comparison to WT controls. Moreover upon APH treatment, the level of phospho-chk1 protein (S345) was significantly lower in ATR seckel ESCs with respect to wild type controls. The reduction of ATR protein in Seckel ESCs was accompanied by the extensive karyotype abnormalities in these cells (Fig 7.10C-D).

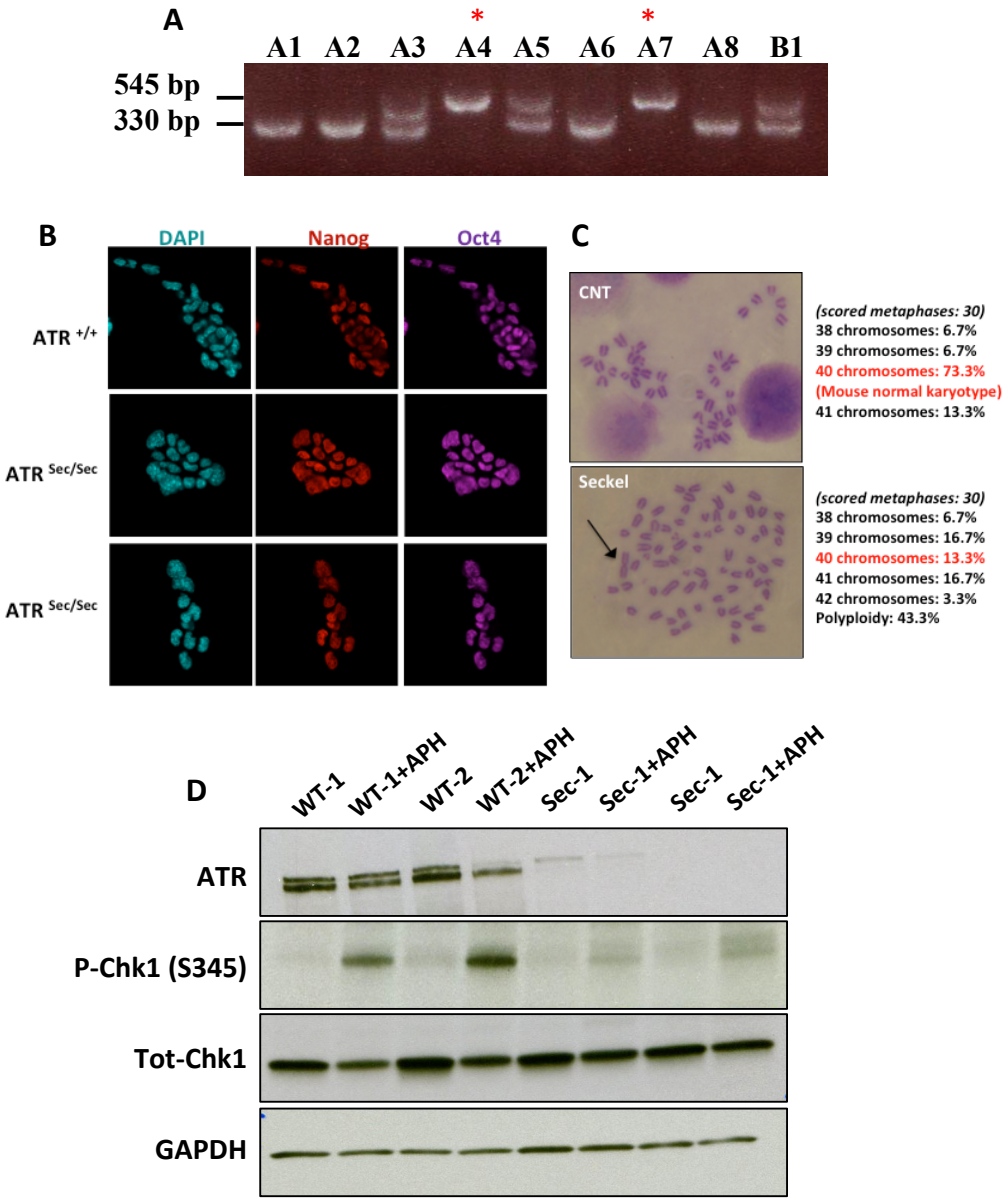


Fig 7.10. Characterization of ATR Seckel ESC.

A) ATR Seckel ESC genotyping. (330bp band=WT, 545bp band=ATR Seckel (*). **B)** Immunostaining for pluripotency markers in WT and ATR Seckel ESCs. **C)** Karyotyping analysis showed extensive aneuploidy in ATR Seckel ESC with respect to wild type cells. **D)** Western blot showing the expression level of ATR and p-Chk1 proteins in two WT and two ATR Seckel lines.

Importantly, the qPCR results in two ATR Seckel and two control lines validated the microarray data for a large panel of 2C-like specific genes, excluding any possible unspecific inhibitory effect of ATR inhibitor on relevant pathways. Of note, similar to inhibitory impact of ATRi on expression of totipotency genes in ESC under normal culture condition, APH treatment on ATR Seckel ESC led to the milder increase of 2C-like genes expression unlike normal wild type ESC (Fig 7.11A-B). These data collectively suggest that activation of ATR-dependent DNA damage response pathway could trigger the global reactivation of totipotency gene network and that ATR inhibition exerts a robust suppressive impact on such transcriptional alterations.

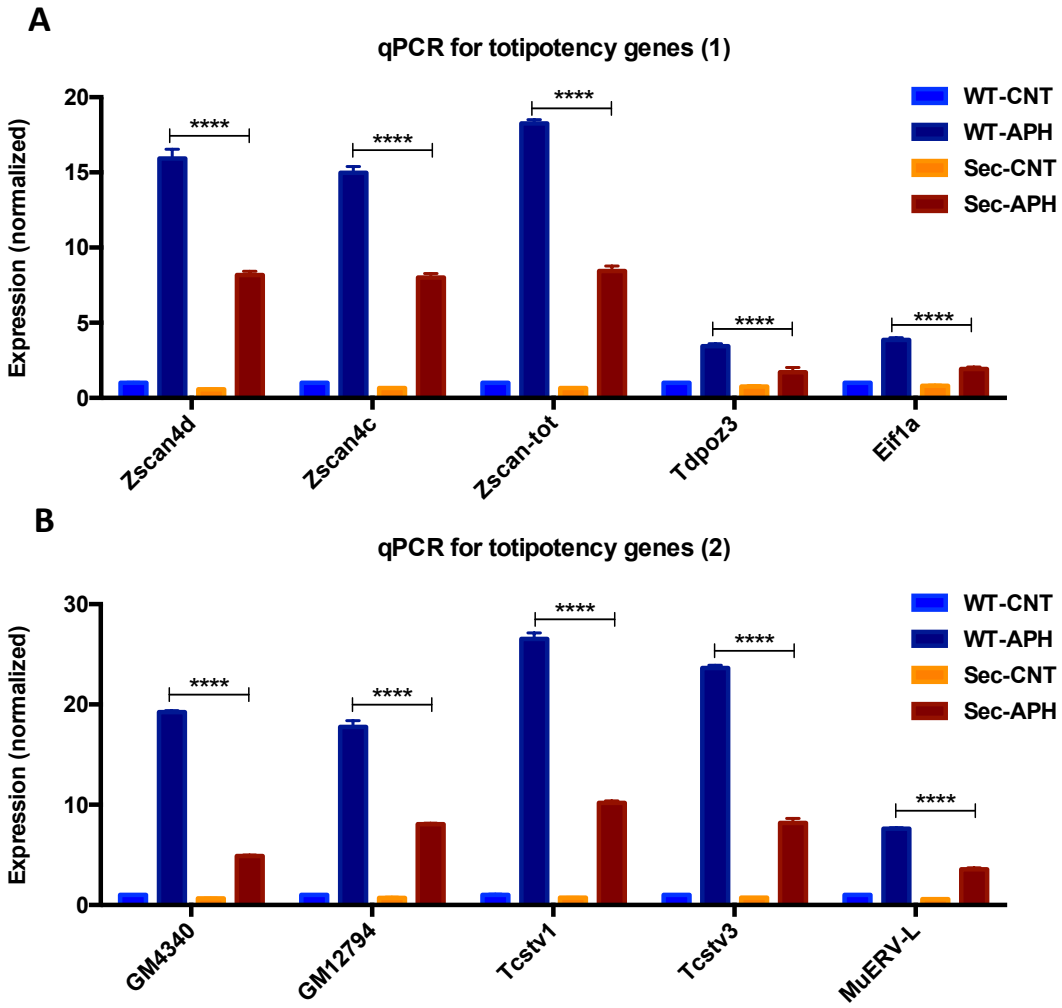


Fig 7.11. Expression of key totipotency genes upon replication stress in ATR Seckel ESC

A,B) qPCR analysis for expression of totipotency genes at mRNA level in ATR Seckel lines with respect to WT control (WT=wild type, Sec=ATR Seckel).

Previous reports demonstrated that 2C-specific genes were increased in mutant ES cells lacking the histone lysine-specific demethylase gene Kdm1a (also known as LSD1), chromatin assembly factor-1 (CAF-1), H3K9 histone methyltranferase G9a, KRAB (Kruppel- associated box)-associated transcriptional repressor Kap1 and ten-eleven translocation (TET) proteins (Macfarlan, Gifford et al. 2012, Maksakova, Thompson et al. 2013, Rowe, Friedli et al. 2013, Lu, Liu et al. 2014, Ishiuchi, Enriquez-Gasca et al. 2015).

Thus, to test whether replication stress could activate the expression of 2C-like genes through interacting with similar pathways, we compared the transcriptome of RSIT-LC with those published datasets. This analysis revealed that overlap of DEGs in RSIT-LC with Caf1 KO cells (23 out of 83 genes) but not with Kap1, Lsd1 or G9a, TET3KO mutant cells. Of note, 48% of the shared DEGs in Caf1 were transcriptionally reverted by addition of ATR inhibitor (data not shown). However, the small number of overlapping genes between RSIT-LC DEGs and those indicated above, collectively suggest an additional mechanism by which replication stress induces expression of totipotency related genes.

7.4. Replication stress increases the transition to 2C-like cells

The flow cytometry analysis on Zscan4-Emeraled ESC showed that the upregulation of Zscan4 is accompanied by increase in the percentage of the Zscan4 positive population suggesting that there is a significantly higher fraction of 2C-like cells with respect to the normal embryonic stem cells in culture after treatment with APH. However, this could be for instance due to changes in division and death rates of Zscan4 positive and negative cells (such as decreased survival of Zscan4-negative cells, or an increase in the division rate of Zscan4-positive cells), rather than alterations in the switching rate between the negative and positive states (Fig 7.13A). Thus, to exclude if the expression of key 2C-like specific genes is due to depletion of Zscan4 negative population, which could lead to the preferential enrichment of the pre-existed Zscan4-positive population upon replication stress, we first evaluated the casapase3 expression in Zscan4 positive and negative population to check if Zscan4 negative population are more prone to cell death with respect to Zscan4 positive cells. Interestingly, in line with higher load of replication stress in Zscan4 positive population under normal culture condition, we found

6-fold increase in the activation of caspase-3 in Zscan4 positive population with respect to Zscan4 negative cells. Importantly, we found a higher ratio of caspase-3 positive cells within zscan4 positive population upon low (0,3uM) and high (6uM) concentration of APH (Fig 7.12), which could exclude the higher susceptibility of Zacan4 negative population to undergo apoptosis upon DNA replication stress. In addition, these results suggest that Zscan4+ cells are more susceptible to undergo apoptosis and it is unlikely that replication stress could selectively enrich the culture in the favor of Zscan4+ population.

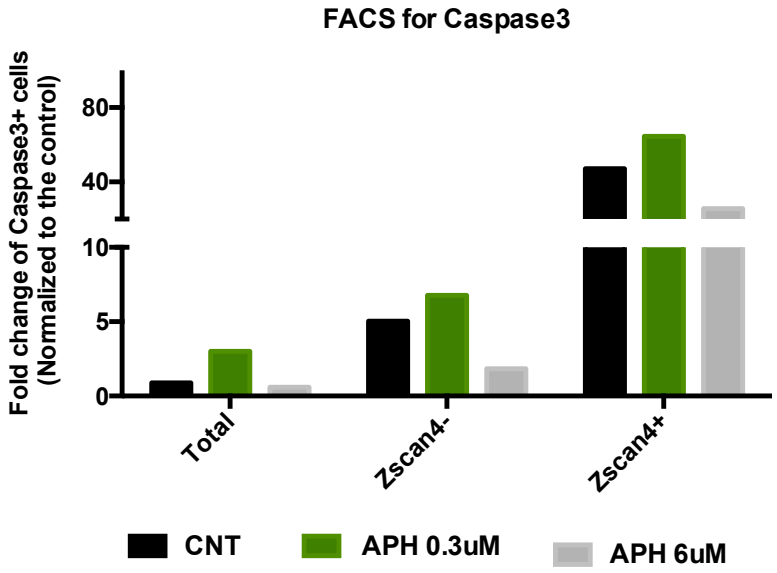


Fig 7.12. FACS analysis for Caspase3 apoptosis marker in Zscan4 positive and negative populations after treatment with APH at two distinct concentrations.

Next, to further consolidate these results, we exposed FACS sorted positive and negative Zscan4 cells to APH. Next, we counted cells every 24 hrs and checked their Zscan4 expression by FACS analysis daily up to 4 days. Therefore, based on the experimental data we employed a theoretical model with stochastic events of cell division, cell death, and switching rates (see Materials and Methods) (Fig 7.13B,C). The rates of

switching between positive and negative states in the control conditions were fixed by fitting the model against the experimental data (Fig 7.13B). Fig 7.13C shows that the null hypothesis in which these rates remain the same in the treated condition requires a large (more than two-fold) increase in the growth rate of APH-treated Zscan4-positive cells with respect to the Zscan4-positive population in untreated condition. In other words, the only condition in which we could exclude the contribution of transition from Z- to Z+ state in enrichment of Zscan4+ population is if Z+ cell divide more that 2.5 fold faster upon APH treatment with respect to Z+ cells in control condition. This corresponds to an extremely unrealistic situation and is not supported by our experimental data. Overall, these results support that APH increase the switching rate of Zscan4 negative population toward Zscan4 positive ones.

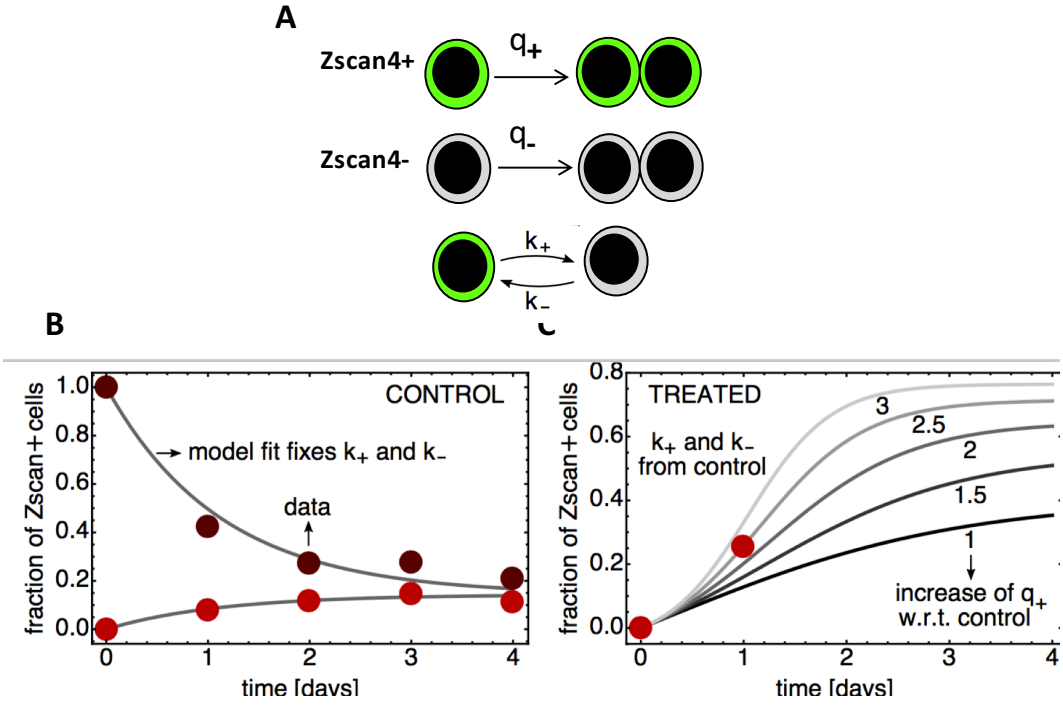


Fig 7.13. Mathematical model with stochastic process of cell division, death, and switching showed that the observed increase in Zscan4-positive cells in APH treated culture is due to the change in switching rates from Z- to Z+ sates.

The model is expressed in terms of four rates: the net growth rate q+ (representing cell division and cell death) of positive cells, the net growth rate q- of negative cells, the switching rates k+ and k-. The net growth rates are equal to a duplication rate minus a death rate for the specific cell type. **B,C)** All model parameters are fixed in the control experiment, where the fraction of positive cells (circles) converges to a long-time value around 5%. The curves are model fits, yielding k+ = 0.9±0.1, k- = 0.14±0.03. Supposing the switching rates remain the same in the treated culture, the experimental data (circles) can be realized in the model only if the net growth rate q+ of positive cells is 2.5±0.5 times larger than in the control, which is highly unrealistic. Different curves are obtained for increasing (from bottom to top) values of q+, starting from the value in the control. The growth rate of negative cells, q-, is fixed by cell counts at t=1d.

Next, in order to understand the impact of transient activation of replication stress response on generating SSB or DSB, I stained ESC with 53BP1 and H2aX, 24 hrs post treatment but also after three passages in fresh media. Although the number of 53BP1 nuclear bodies increased up to 3 fold 24 hrs post APH treatment, I did not find any significant change of that after 3 passages of ESC (Fig 7.14A and B) suggesting that the transient activation of replication stress response by APH does not lead to permanent lesion on ESC genome probably due to the activation of DNA repair genes. Importantly, I also did not observe any significant alteration in the number of co-localized H2AX and 53PB1 nuclear bodies suggesting that transient treatment with APH does not result in DSB formation in ESC (Fig 7.14C and D).

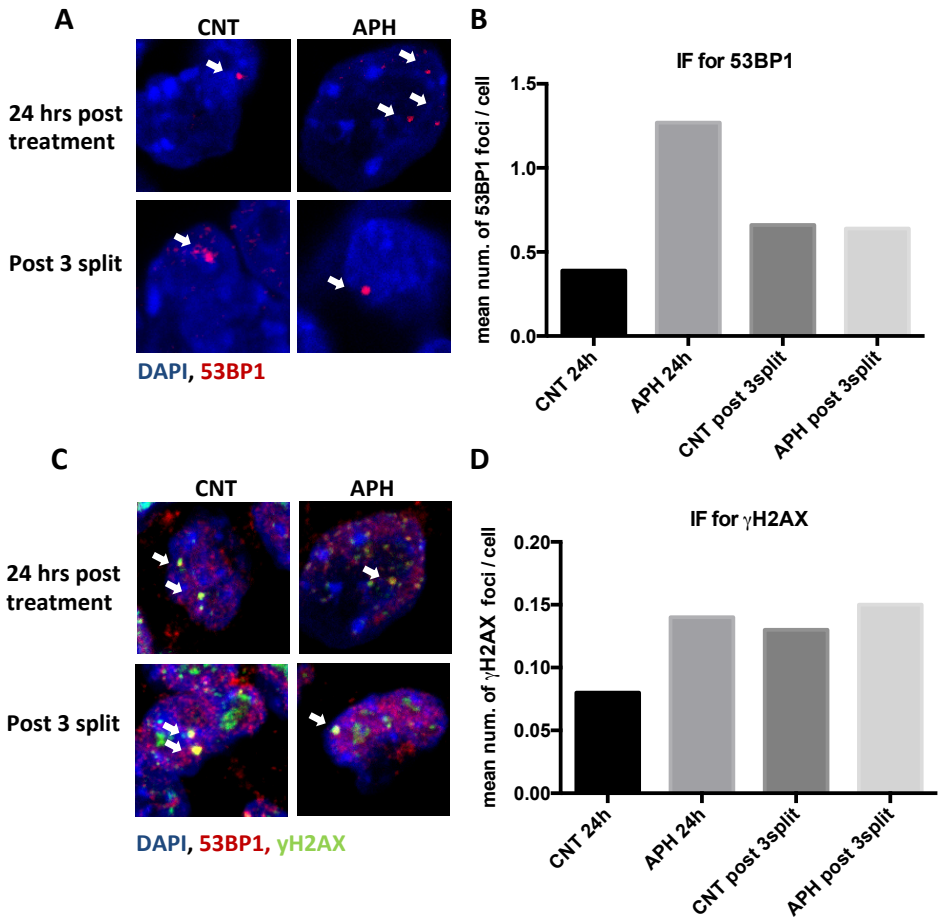


Fig 7.14. Immunostaining for γH2AX and 53BP1 foci in ESC.

A-D) Immunofluorescence showing 53BP1 and γH2AX nuclear bodies in APH treated ESC versus control 24 hrs post treatment and after three passages. The bar plots representing the average number of 53BP1 and γH2AX nuclear body per cell in each condition (n=10 field/condition).

7.5. ATR activated 2C-like cells gain expanded developmental potential

Our results so far indicated that ATR activated ESC exhibit similar transcriptional profile and characteristics of 2-cell-stage embryos. Strikingly, computational gene-sets analysis of APH vs CNT through molecular signature database (MSigDB), identified a strong and highly significant enrichment in placenta-related pathways which are robustly suppressed upon ATR inhibition (Fig 7.15A).

In addition, gene expression results also revealed that the activation of some key placenta related genes upon replication stress in PSC including Fstl3, Dgka, Egfr, GPX3, Slc7a8, Eng, Cyr61, Adm, Scn1b, Emp3, Anpep, Cryab and Hspb2. some of which are interestingly involved in exosome formation and angiogenesis (such as Dgka, Eng or Egfr), the critical pathways that are potentially exploited by placenta to gain migratory, invasive and immune suppressive features (Fig 7.15B)

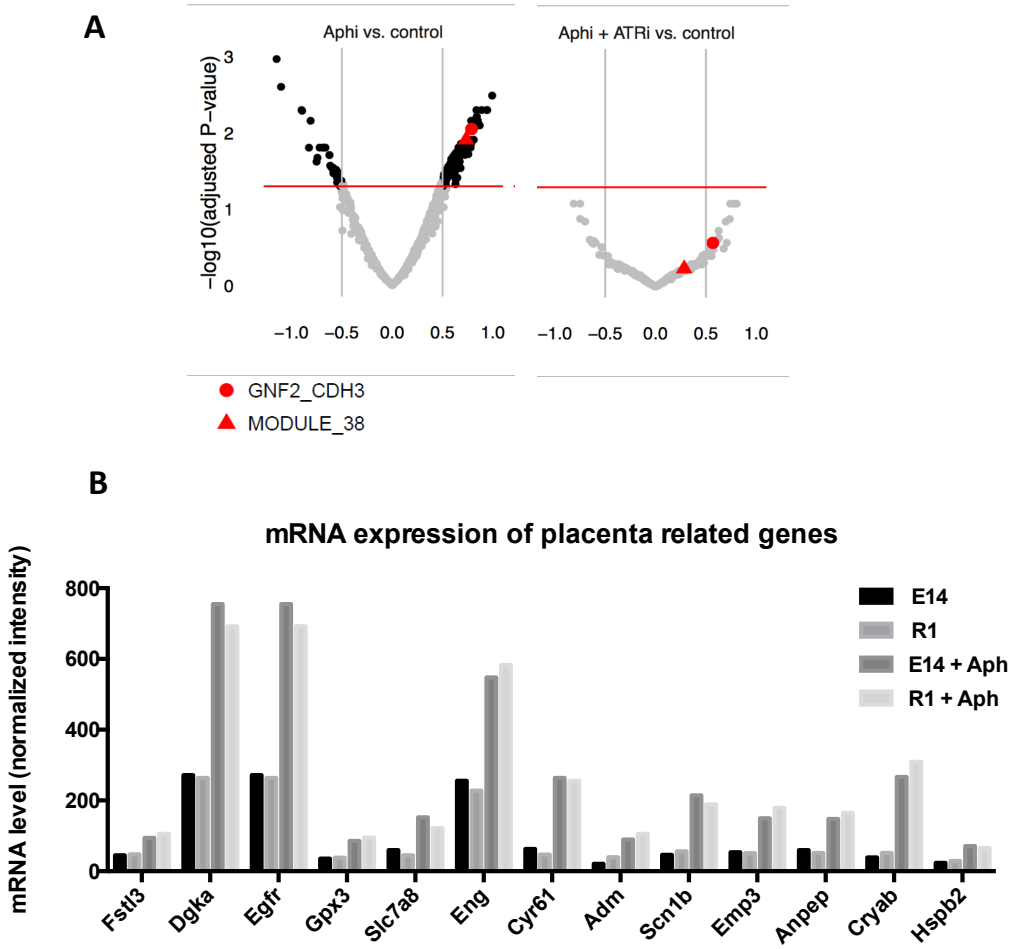


Fig 7.15. A global activation of placenta related gene categories upon APH treatment.

A) The Volcano plot shows a strong and highly significant upregulation of the placenta related gene categories upon APH treatment while ATR inhibitor reverted them back to the control level (all the gene categories above red line are significant and the categories upregulated are in the right part of the panel, while the gene categories on the left are

downregulated). **B)** Expression of placenta related genes in E14 and R1 ESC after treatment with APH at mRNA level.

These results overall suggest that RSIT-LC have gained expended development potency and thus are more prone to be differentiated to extra-embryonic tissues. Hence, we asked if ATR activation could promote differentiation toward trophectoderm lineage. To this aim, both ATR Seckel and control ESC cells (upon treatment with APH) were differentiated *in vitro* toward trophoblast-like stem cells for three days followed by further differentiation to giant trophoblast -like cells upon withdrawal of FGF4 and heparin for 3 additional days (see Material and Method) (Abad, Mosteiro et al. 2013). Interestingly, along with higher number of differentiated cells in APH treated control cells, qPCR results revealed the highest expression of main trophoblast stem cell specific marker "Cdx2" in ATR proficient condition while ATR Seckel cells did not significantly upregulate this marker in the absence of APH (Fig 7.16).

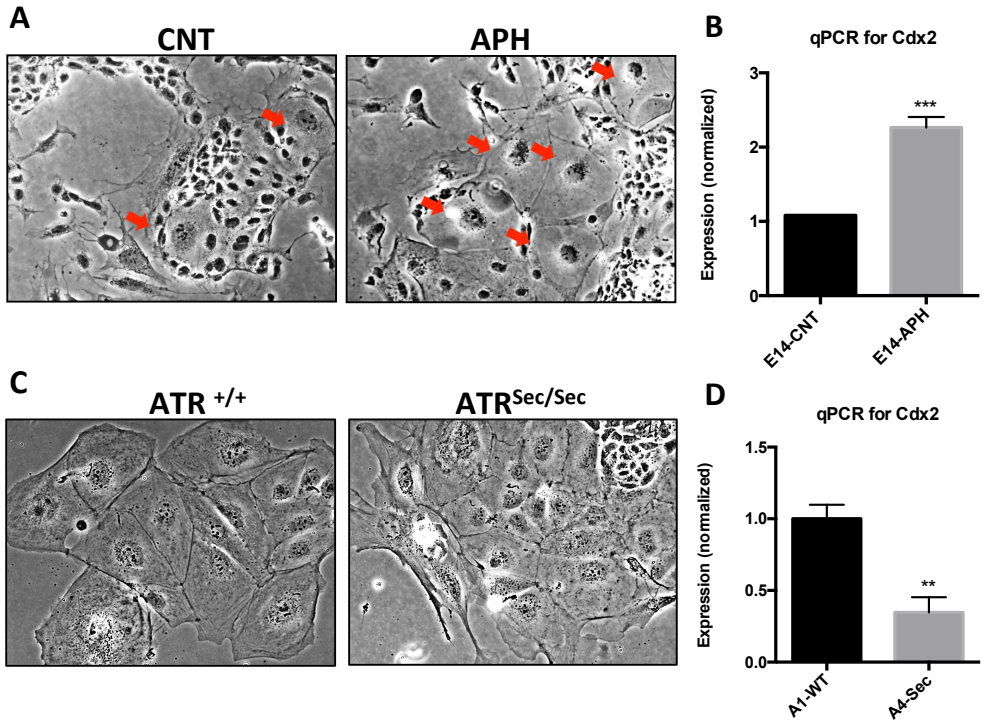


Fig 7.16. Replication stress induced 2C-like cells gain expanded *in vitro* developmental potential.

A-D) Phase contrast images and qPCR results of E14-WT and ATR Seckel ESC upon aphidicolin treatment. qPCR results revealed activation of trophoblast specific marker, Cdx2 upon activation of ATR. **D)** Cdx2 expression is significantly hampered upon differentiation of ATR Seckel ESC toward giant trophoblast-like cells with respect to WT ESC.

Next, to understand if these cells have also gained distinct functional characteristics to give rise to extra-embryonic tissues along with inner cell mass derived lineages, Zscan4-emerald ESC were infected with a lentivirus encoding mCherry protein (from constitutively active EF1a-promoter (which is reported not to be silenced in ESC unlike CMV promoter), sorted for mCherry expression and subsequently treated with APH. Next, we injected five to eight cells into morula-stage embryos taking advantage of laser assisted microinjection technique (experimental set up is shown in Fig 7.17A) and 24 hrs post injection the contribution of cells into inner cell mass and trophectoderm layer were analyzed (Fig 7.17B). Interestingly, in 7 out of 35 (20%) chimeric embryos, APH-induced 2C-like cells contributed to both ICM but also the trophectoderm layer of the blastocysts, while none of the un-treated CNT cells could contribute to the trophectoderm lineage (Fig 7.17B). This results suggest that the developmental potential of ATR activated cells includes embryonic plus extra-embryonic tissues in contrast to normal ESC, which are mostly restricted to generate embryonic cell types.

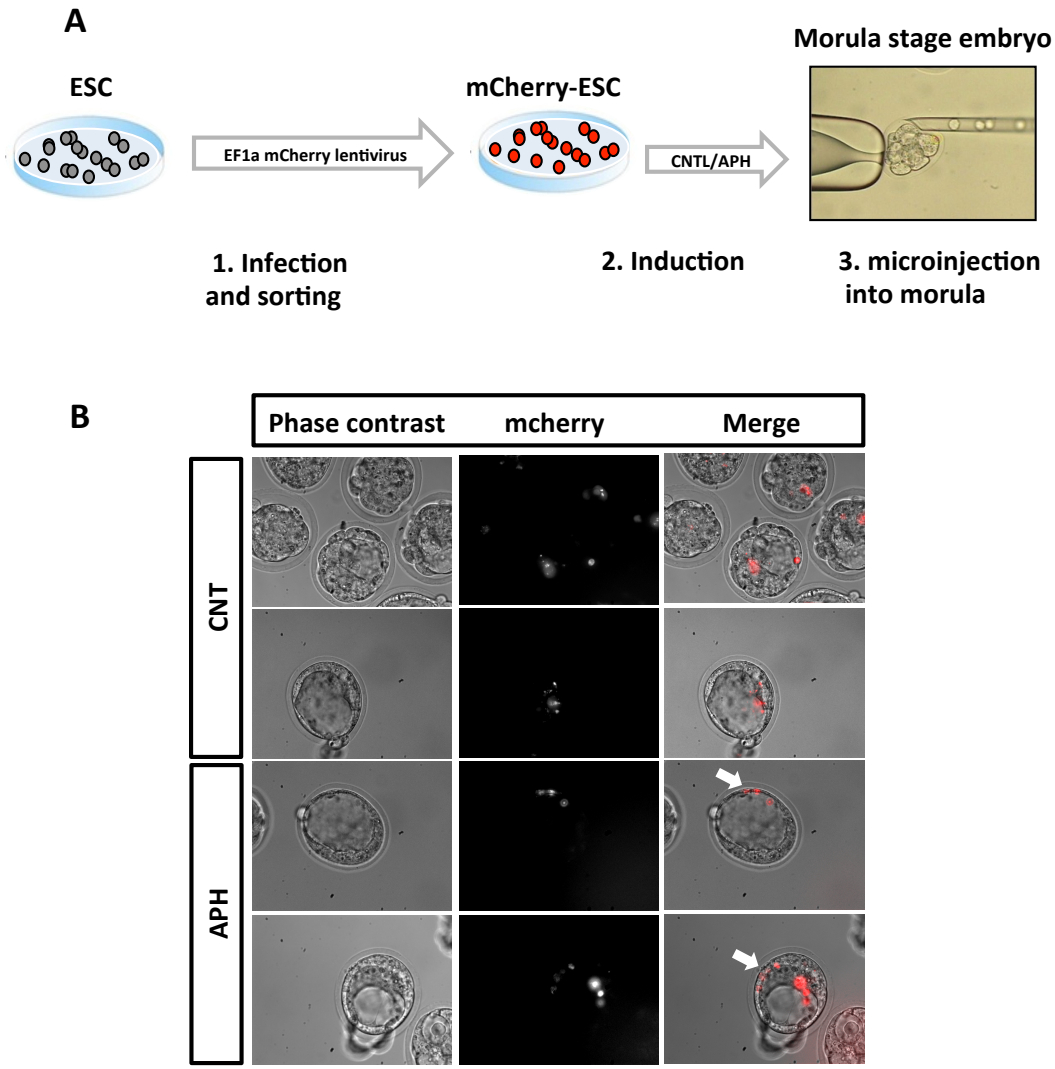


Fig 7.17. Replication stress induced 2C-like cells gain expanded *in vivo* developmental potential.

A) A scheme showing the experimental setup for laser-assisted cell injection into morula stage. **B)** APH-treated cells contributed to the ICM and trophectoderm layer of the blastocysts unlike normal ESC.

7.6. Somatic cell reprogramming in the presence of Cdc45

Although reprogramming to pluripotent state has been demonstrated by Yamanaka in 2006, to our knowledge, no data on *in vitro* reprogramming of somatic cells to totipotent-like state is reported yet. In order to understand if a mild activation of DNA replication stress response during reprogramming could elevate the expression of key totipotency genes in emerging iPSC colonies, we searched for the replication factors that are known to induce replication stress when they are overexpressed. We chose Cdc45, an essential replication initiation factor and Cdc6, one of the key components of pre-replicative complex as their over-expression have been shown to induce replication stress previously (Bartkova, Rezaei et al. 2006, Srinivasan, Dominguez-Sola et al. 2013). Prior to test the effects of these replication factors during reprogramming, we examined the impact of their over-expression in E14 cells. To this aim, Cdc45 and Cdc6 cDNA were cloned into a Flag-tagged lentivector with EF1α promoter and then E14 cells were infected and subsequently underwent puromycin selection step (the overexpression of Cdc45 and Cdc6 were confirmed by western blot, fig 7.18A). Although Cdc45 and Cdc6 over-expressing ESCs maintained their typical morphology in culture (Fig 7.18B), qPCR assay revealed that the expression of the key two-cell embryo-specific genes including Zscan4, Gm12794, Gm4340 and MuERV-L are upregulated in Cdc45 and Cdc6 infected cells versus empty vector (EV) infected control condition (Fig 7.18C,D). However, overexpression of these factors on mouse embryonic fibroblast (MEF) did not alter the expression of MuERV-L gene (Zscan4d was not expressed in MEF cells), suggesting the specific role of these genes in pluripotent stem cells (Fig 7.18E).

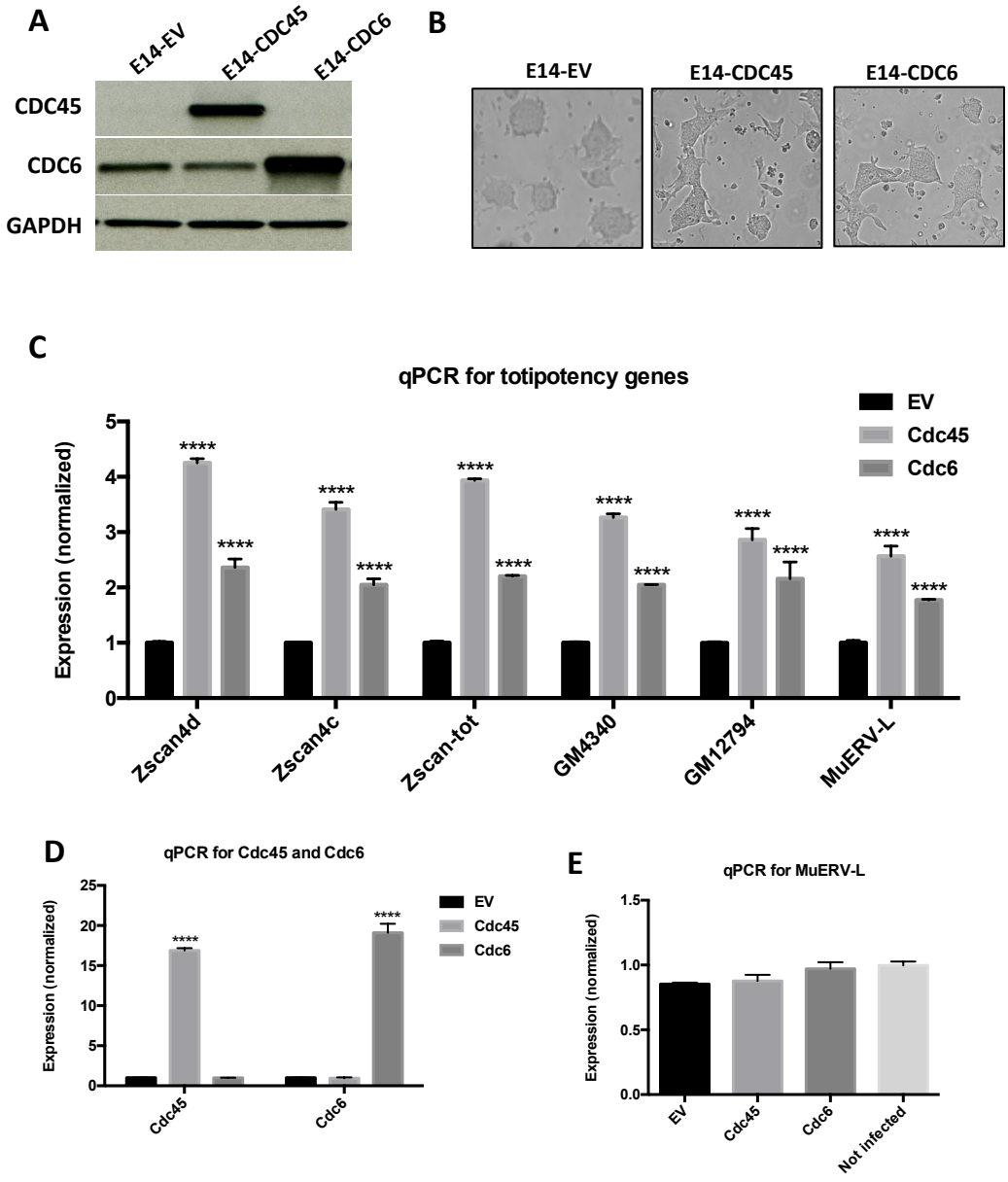
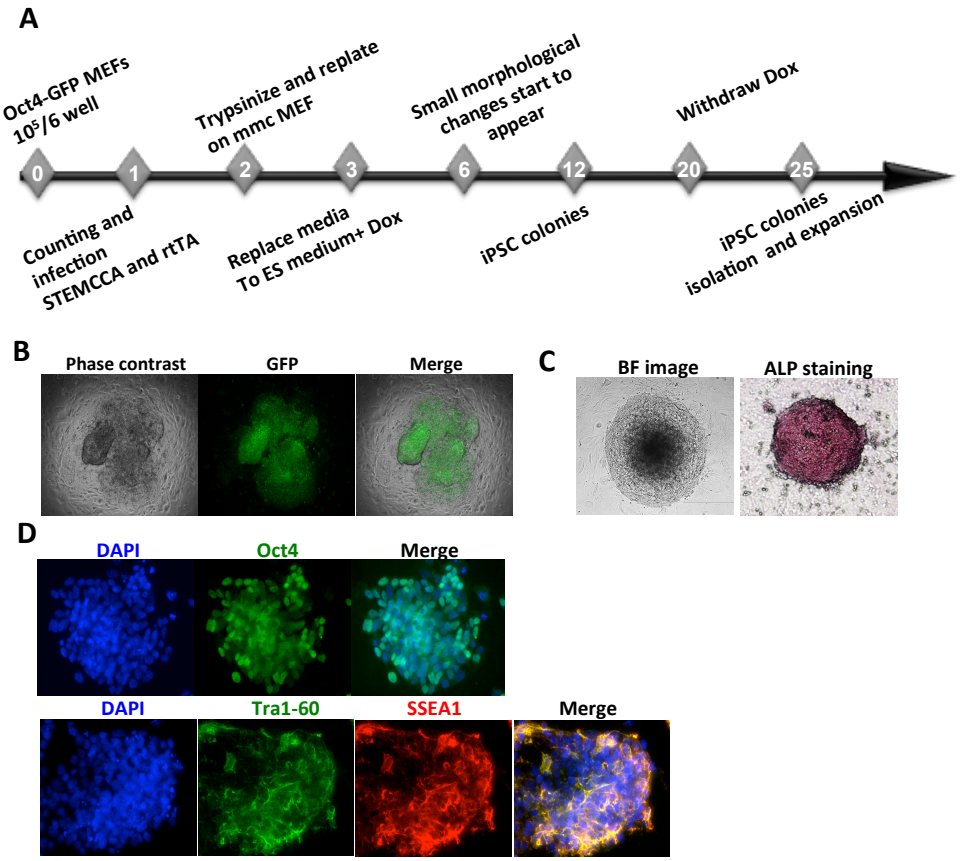


Fig 7.18. Overexpression of Cdc45 and Cdc6 elevated the expression of 2C-specific genes in E14 ESC line.

A) Western blot for Cdc45 and Cdc6 on E14 cells after infection **B)** Bright field images of Cdc45, Cdc6 overexpressing E14 cells **C)** qPCR assay for some key totipotency genes on Cdc45, Cdc6 over expressing E14 cells. **D)** Expression level of Cdc45 and Cdc6 in infected ESC. **E)** mRNA expression level of MuERV-L in MEF cells under empty vector (EV) control, Cdc45 and Cdc6 overexpressing conditions (EV=empty vector control).

Next, we asked if over-expression of Cdc45 during reprogramming could further reprogram the somatic cells back to the earlier embryonic stages. To this aim, we first established somatic cell reprogramming (Fig 7.19A) using low passage Oct4-GFP MEFs that are expressing GFP under the control of Oct4 promoter and upon acquisition of pluripotency cells turn in to green as it is depicted in fig 7.19B. The picked colonies were positively stained for ALP and other pluripotency markers such as Oct4, SSEA1 and Tra1-60 (Fig 7.19C-D).

Next, we co-infected Cdc45 with reprogramming cocktail (Oct4, Sox2, c-Myc and Klf4) and followed the established reprogramming protocol. Upon withdrawal of doxycycline *bona fide* iPSC colonies were picked and examined to identify colonies harboring Cdc45 expressing vector. Out of twelve colonies, we could identify two clones (Cdc45-3 and Cdc45-9), which had a higher level of Cdc45 both at protein and mRNA level (Fig 7.19E and 7.21A). These results were also confirmed by blotting for anti-Flag antibody (Fig 7.19F).



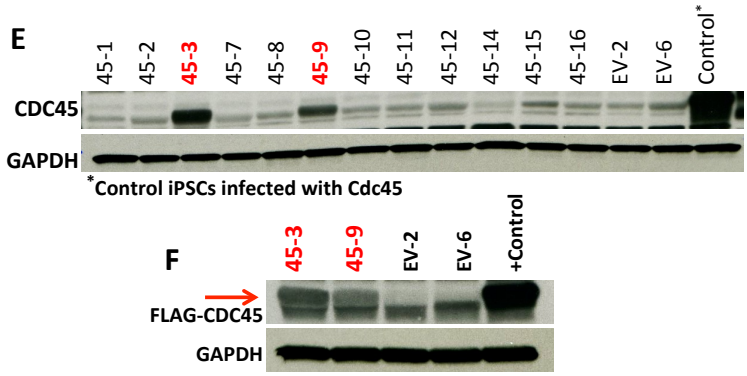


Fig 7.19. Activation of mild replication stress during somatic cell reprogramming resulted in elevation of totipotency genes in iPSC colonies.

A,B) Schematic view of somatic cell reprogramming protocol **d)** Expression of endogenous GFP upon reprogramming of Oct4-GFP MEFs. **C,D)** Reprogrammed colonies are positively stained for ALP and pluripotency markers. **E,F)** Identification and verification of Cdc45 over-expressing iPSC colonies**.**

As shown in Fig 7.20A besides maintenance of typical iPSC morphology, Cdc45 induced iPSC colonies were positively stained for key pluripotency markers such as ALP, Oct4, Nanog and SSEA1. Moreover, qPCR results confirmed that Cdc45 overexpressing iPSC express pluripotency genes quite at the homogenous level (Fig 7.20C). Next, to exclude the possibility that Cdc45 induction favors a certain cell lineage commitment, we performed *in-vitro* differentiation assay through the formation of embryoid bodies. These results showed that Cdc45-iPSC are able to contribute to the three main embryonic lineages namely endoderm, mesoderm and ectoderm by activation of key lineage specific transcriptional factors similar to the control iPSC. This activation was also accompanied by the robust repression of key pluripotency genes upon differentiation (Fig 7.20 B-D).

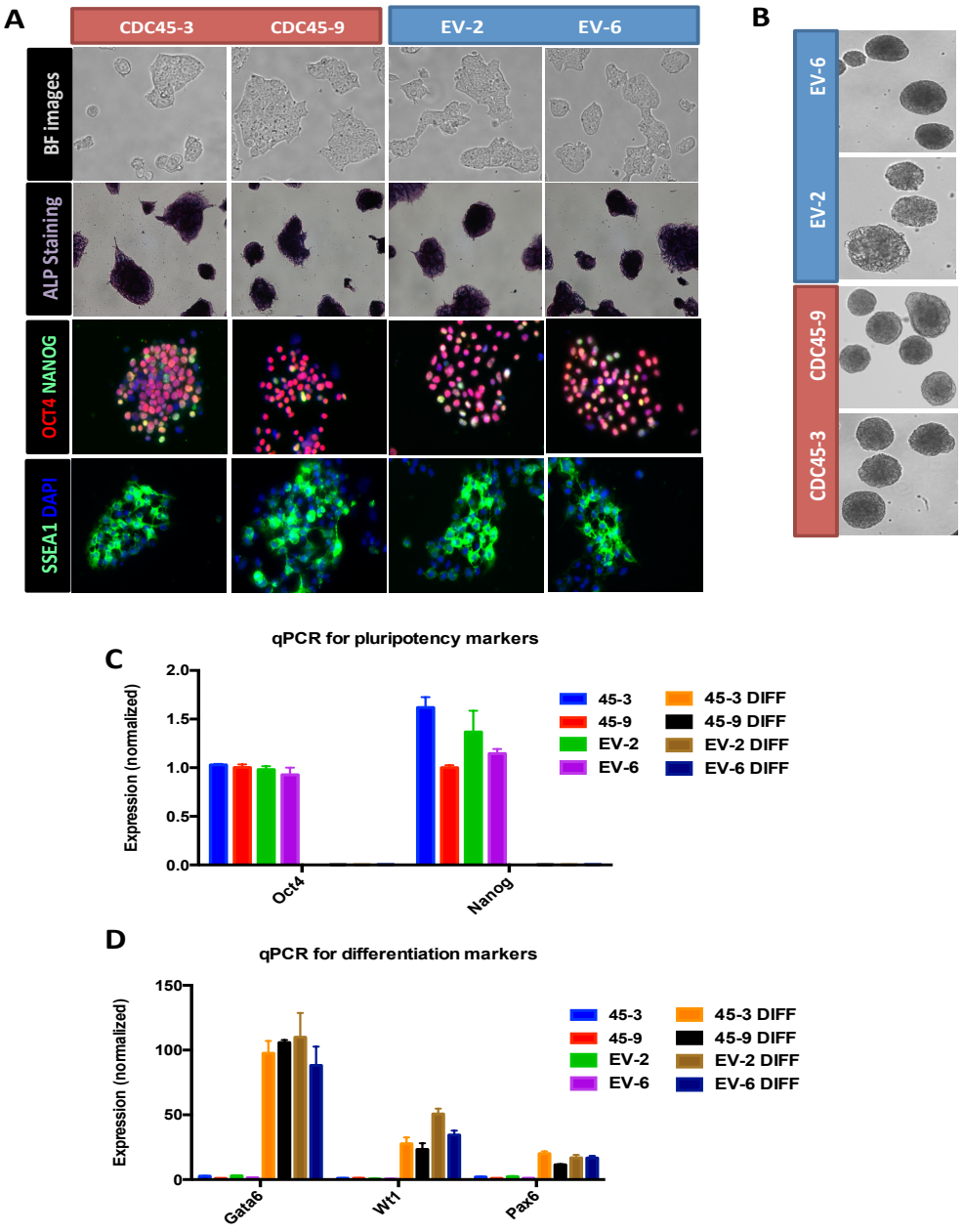


Fig 7.20. Characterization of Cdc45 overexpressing iPSC.

A) Immunofluorescence for pluripotency markers on control and Cdc45 overexpressing iPSC colonies. **B)** In-vitro differentiation of control and Cdc45 overexpressing iPSC colonies to embryoid bodies **C)** qPCR assay results showed that pluripotency markers were silenced upon induction of differentiation in all iPSC clones **D)** qPCR for endoderm (Gata6), ectoderm (Pax6) and mesoderm (Wt1) differentiation markers on control and Cdc45 over expressing colonies.

Then we performed qPCR on Cdc45 overexpressing colonies for some of totipotency specific genes where the results not only demonstrated upregulation of some key 2C-like genes in Cdc45 overexpressing iPSC, but more importantly revealed that there is a Cdc45 dosage dependent gene expression pattern for some of the key 2C-like specific genes such as MuERV-L, Zscan4 and GM12794 (Fig 7.21) (Macfarlan, Gifford et al. 2012, Ishiuchi, Enriquez-Gasca et al. 2015). These results overall suggest that Cdc45 not only induce expression of totipotency genes on already established PSCs but its expression could also generate iPSC which express 2C-like specific genes.

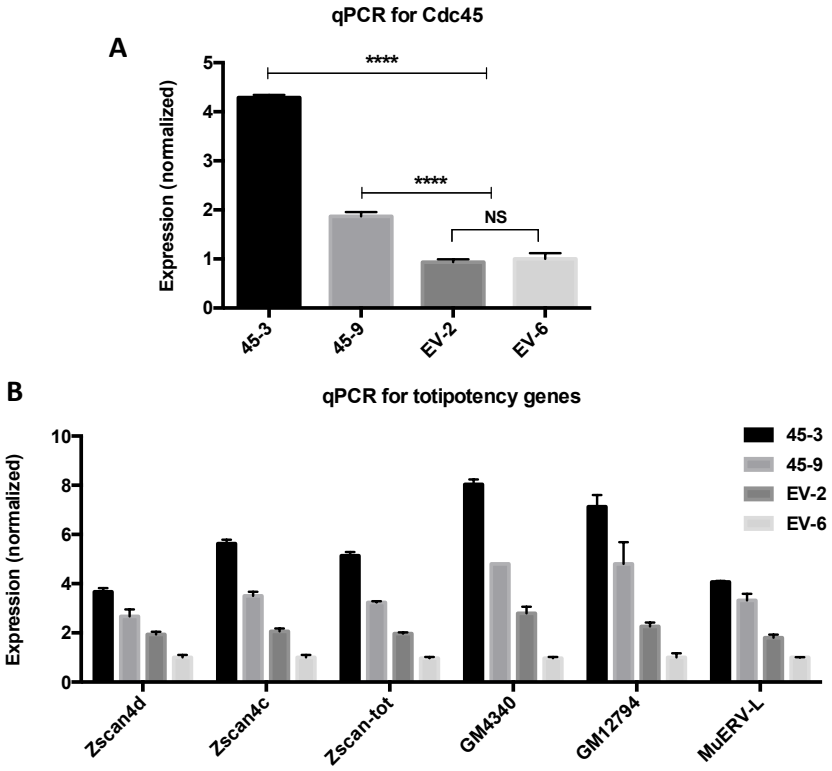


Fig 7.21. A, B) qPCR results for key totipotency genes in Cdc45 overexpressing colonies.

8. Discussion

Several studies show that mESC exhibit high level of γH2AX in the absence of measurable numbers of DNA double-strand breaks. Importantly, the high numbers of endogenous γH2AX foci in pluripotent mES cells was found to be dependent on the activity of the apical checkpoint kinase ATM or DNA-PK and notably, is not associated with the double- strand break (DSB) marker 53BP1 (Ziegler-Birling, Helmrich et al. 2009).

A recent interesting study from Lopes lab, shows that H2AX phosphorylation in cultured ESCs and mouse embryos is instead dependent on Ataxia telangiectasia and Rad3 related (ATR) and is associated with chromatin loading of the ssDNA-binding proteins RPA and RAD51. Moreover, their results reveal that ESCs display massive accumulation of ssDNA gaps, reduced fork speed and frequent fork reversal through single-molecule analysis of replication intermediates. Interestingly, they have found that delaying the G1/S transition in ESCs allows formation of 53BP1 nuclear bodies and suppresses ssDNA accumulation, fork slowing and reversal in the following S-phase suggesting that a short G1 phase imposes constitutive replication stress and fork remodeling in mouse embryonic stem cells (Ahuja, Jodkowska et al. 2016).

Despite of reported constitutive DNA replication stress in ESC, the mutation frequency in ES cells is significantly lower than that in mouse embryonic fibroblasts This could be since ESC are critical for embryo formation as they ensure the lifetime maintenance of any tissues upon differentiation. Thus, any misrepair of DNA damage in ESC can be transmitted to multiple cell lineages, thereby compromises tissue integrity and function. That is why ESC has to come up with several modifications in DNA damage response, which accommodate dynamic cycling and preservation of genetic information. For instance, mESC mostly utilize the extremely precise repair mechanism i.e. HRR to

repair their DNA with respect to differentiated cells that use NHEJ (Lin, Chao et al. 2005, Tichy and Stambrook 2008, Serrano, Liang et al. 2011, Schneider, Pellegatta et al. 2013, Santos, Faryabi et al. 2014).

Interestingly, the exceptional genomic stability of mESC has been linked to Zscan4, which has been discovered as a specific marker for two-cell embryo and 2C-like ES cells. Of note, about 5% of ES cells express Zscan4 at a given time, but nearly all ES cells activate Zscan4 at least once during nine passages that is associated with rapid telomere extension through telomere recombination and upregulation of meiosis-specific homologous recombination genes. On the other hand, Zscan4 knockdown ESC are found to have shorter telomeres, increased karyotype abnormalities and spontaneous sister chromatid exchange, which is also accompanied by slowing down cell proliferation until reaching crisis by passage eight. Overall, these findings show a unique mode of genome maintenance in ES cells (Zalzman, Falco et al. 2010, Amano, Hirata et al. 2013).

Here, my results show that expression of Zscan4 is regulated by ATR, a main component of DNA damage response pathway, which is activated upon DNA replication stress. Importantly, further activation of replication stress response via agents such as APH, HU and UV that challenge accurate DNA replication could elevate the expression of Zscan4 in a time and concentration dependent manner. Importantly, activation of Zscan4 was reverted back to the basal level upon addition of ATR and CHK1 inhibitors. These results overall provide a new mechanism through which ESC copes with constitutive replication stress to maintain its genomic stability.

As all living organisms are constantly exposed to genotoxic stress, their genome needs to be repaired to preserve the vital information. DNA damage induces a robust response mediated by the DNA damage repair network at the center of which there are

ATM and ATR proteins, which sense DNA double strand breaks (DSBs) and DNA single-strand breaks (SSBs) to recruit proteins operating in the repair systems. DDR-mediated cellular outcomes may range from cell death by apoptosis, transient cell cycle arrest followed by repair of DNA damage; to cellular senescence caused by the persistence of unrepaired DNA damage (Sulli, Di Micco et al. 2012).

In response to genotoxic stress and/or inappropriate microenvironment, mESCs undergo either apoptosis or differentiation, thus removing damaged cells from the pluripotent pool and eliminating any cell at risk of malignant transformation, while somatic cells undergo apoptosis or cellular senescence to limit the risk of malignant transformation (Tichy and Stambrook 2008, Sulli, Di Micco et al. 2012).

Though DNA damage induced cellular differentiation and apoptosis has been frequently reported in stem cells, To our knowledge, here for first time we report that replication stress in ESC, could lead to the transition to more primitive totipotent-like state followed by the global activation to 2C-like transcriptional network (Schneider, Pellegatta et al. 2013, Santos, Faryabi et al. 2014). More importantly, we found that the activation of totipotency related genes is mediated by ATR, the key components of DDR pathways whose specific inhibition, represses the reactivation of 2C-like transcriptional profile under normal condition but also in the presence of replication stress inducing agents.

Aneuploidy is commonly seen in human pre-implantation embryos, most particularly at the cleavage stage. However, some mosaicisms, such as copy number variations could be compatible with live birth. For example, healthy euploid newborns have been recently reported upon intrauterine transfer of mosaic aneuploid blastocysts pointing to presence of some self-correction mechanisms to repair aneuploidies (Bazrgar, Gourabi et al. 2013, Greco, Minasi et al. 2015). Several explanations have been proposed

for self-correction of aneuploidies during embryonic development, including allocation of the aneuploidy in the trophectoderm, cell growth advantage of diploid cells in mosaic embryos, lagging of aneuploid cell division, and the abundance of DNA repair gene products (Bazrgar, Gourabi et al. 2013). Of note, in 2015 Ko lab reported evidences for Zscan4 role in correction of chromosomal abnormalities in human fibroblast samples from Dawn and Edwards syndrome individuals (though the underlying mechanism is not shown) suggesting that ZGA in particular activation of Zscan4 event could be vital to keep the early embryonic cell genome intact (Amano, Jeffries et al. 2015, Ko 2016).

Consistent with these evidences, my findings show that activation of DNA damage response pathways reprograms the PSC to more primitive and developmentally potent stage by activation of totipotency gene network. Strikingly, I found that replication stress induced ESC, activated global transcriptional network of placenta related genes in an ATR dependent manner. In support of this, higher efficiency in differentiation toward giant trophoblast-like cells was found upon activation of replication stress response. Finally, replication stress induced ESC found to contribute more efficiently to trophectoderm layer of blastocysts upon microinjection into early morula in comparison with CNT ESC. These results overall suggest that replication stress increase the transition of ESC to 2C-like state which is highly enriched for specific DNA repair proteins such as Zscan4 but also activates trophectoderm differentiation program to sequester cells to the extra-embryonic compartment (Zalzman, Falco et al. 2010, Amano, Jeffries et al. 2015) and thus minimize the contribution of the damaged cell to embryos whole body mass.

The critical involvement of ATR to activation of totipotency network but also trophoblast differentiation, were confirmed in ATR Seckel ESC. Besides, validating the specificity of the ATR inhibitor used in the study, our findings shed light on the possible

mechanism that could lead to the premature aging phenotype in ATR Seckel mice as reported by Murga et al. In support of our finding, authors have reported accumulation of necrotic areas and overall loss of cellularity in mutant placentas that could also contribute to the dwarf phenotype regardless of intrinsic developmental defects (Murga, Bunting et al. 2009).

Although reprogramming to pluripotent state has been demonstrated by Yamanaka in 2006, to my knowledge, no data on *in-vitro* reprogramming somatic cells to totipotent-like cells is reported yet. In order to understand if the mild activation of replication stress response could reprogram the somatic cells directly to totipotent-like cells, first I looked for factors that upon over-expression could activate replication stress response in ESC as somatic cell reprogramming in the presence of agents such as APH or HU in long term is not practically possible.

Here, for the first time, I found that overexpression of some factors which are previously shown to activate replication stress response, such as Cdc45/6, not only elevate the expression of totipotency genes on established ESC but also Cdc45 overexpression could elevate the expression of 2C-like genes in emerging iPSC. Of note, I did not observe any atypical tendency in self-renewal or differentiation potency of Cdc45 overexpressing iPSC as they expressed canonical pluripotency markers and directed toward three main embryonic germ layers upon differentiation. Yet, my finding showed that Cdc45 overexpressing colonies elevate the expression of several members of Zscan4 gene family, which are associated with genomic stability of PSC. However, additional experiments are needed to understand if these factors are exerting their effect through similar mechanism of replication stress inducing agents.

In conclusion, our findings not only shed light on exogenous and endogenous stimuli that could contribute to the genome plasticity of PSC but also provide an important insight on the critical mechanisms that PSC exploit to cope with genotoxic stress.

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