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Netrin-1 function in somatic cell reprogramming and pluripotency

Duygu Ozmadenci

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**Netrin-1 function in somatic cell
reprogramming and pluripotency**

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“Learning is a process where knowledge is presented to us, then shaped through understanding, discussion and reflection“

“Banking education treats students as objects of assistance; problem posing education makes them critical thinkers”

Paulo Freire

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ABSTRACT

Pluripotency is the ability of embryonic epiblast cells to self-renew and to give rise to all somatic cells as well as germ cells. Somatic cells can also be reprogrammed toward pluripotency, opening new avenues for stem cell based therapies in the treatment of degenerative diseases. Deciphering the molecular mechanisms, and in particular signaling pathways that control pluripotency is crucial to improve our understanding of early embryogenesis and the use of iPSC (inducible Pluripotent Stem Cell) in regenerative medicine.

Herein, I provide the first description of Netrin-1 as a regulator of reprogramming and pluripotency. Netrin-1 and its receptors are present in many cell types and are engaged in a variety of cellular processes beyond its initial characterization in the neuronal system. In the first part, I contributed to explore how Netrin-1 prevents apoptosis mediated by its dependence receptor DCC (Deleted in Colon Carcinoma) during reprogramming. In the second part, I dissected the functions and regulation of this pathway in pluripotency maintenance and in lineage commitment.

Key words: pluripotency, reprogramming, lineage commitment, Netrin-1, Dependence Receptor

RESUME

La pluripotence est la capacité d'une cellule à s'auto-renouveler et à donner toutes les cellules somatiques ainsi que les cellules germinales. Les cellules pluripotentes peuvent être aussi reprogrammées à partir de cellules somatiques, ouvrant ainsi de nouvelles opportunités pour l'utilisation thérapeutique des cellules souches dans le traitement des maladies dégénératives. La connaissance des mécanismes moléculaires, en particulier des voies de signalisation qui contrôlent la pluripotence, est cruciale pour l'amélioration de notre compréhension de l'embryogenèse précoce et l'utilisation des iPSC (cellules souches pluripotentes induites) dans la médecine régénérative.

Ici, je donne la première description de la Nétrine-1 en tant que régulateur de la reprogrammation et de la pluripotence. La Nétrine-1 et ses récepteurs ont été initialement caractérisés dans le système neuronal, mais il a aussi été montré qu'ils étaient exprimés dans différents types cellulaires et impliqués dans divers processus. Dans la première partie, j'ai contribué à explorer comment Nétrine-1 empêche l'apoptose médiée par son récepteur à dépendance DCC (Deleted in Colon Carcinoma) pendant la reprogrammation. Dans la deuxième partie, j'ai disséqué les fonctions et la régulation de cette voie dans le maintien de la pluripotence et dans l'engagement des lignages.

Mots clés : pluripotence, reprogrammation, engagement des lignages, Nétrine-1, Récepteur à Dépendance.

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ABBREVIATION LIST

AA	Ascorbic Acid
APP	β -amyloid precursor protein
BMP	Bone Morphogenic Protein
CAM	Cell Adhesion Molecule
cAMP	cyclic adenosine monophosphate
CaMKII	calmodulin-dependent protein kinase II
CaN	calcineurin
Cdx2	Caudal Type Homeobox 2
Cer	Cerberus
CH	CHIR99021
DAG	diacylglycerol
DAPK	Death-Associated Protein Kinase
DCC	deleted in colorectal carcinoma
DD	death domain
DiM	(S)-(+)-dimethindene maleate
DIP13 α	DCC-interacting protein 13- α
DOR	Dorsomorphin
DOX	Doxycyclin
Dpc	days post coitum
DR	Dependence Receptor
DSCAM	The Down syndrome cell adhesion molecule
ECATs	ES cell-associated transcripts
EC	embryonal carcinoma
ECD	ectodomain
ESC	embryonic stem cells
EB	embryoid body
EGF	Epidermal Growth Factor
ENA/VASP	Enabled/vasodilator-stimulated phosphoprotein
EpiLC	epiblast-like cells
EpiSC	epiblast stem cell
EPS	extended pluripotent stem
ERK	Extracellular Regulated Kinase
Esrrb	Estrogen Related Receptor Beta
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor 4
FK	Forskolin
FLRT	Fibronectin Leucine Rich Transmembrane Protein
FN	fibronectin
Foxa2	Forkhead Box A2
Foxp1	Forkhead Box P1
GSK3	Glycogen Synthase Kinase 3
iPSC	induced pluripotent stem cell
ICM	inner cell masses

Id	inhibitor of differentiation
Ig	immunoglobulin
IKK	I κ B Kinase
IP3	inositol 1,4,5-triphosphate
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
KLF	Kruppel Like Factor
KSR	Knockout Serum Replacement
LCCs	L-type calcium channels
Lef1	Lymphoid Enhancer Binding Factor-1
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
lincRNA	intergenic non-coding RNA
lncRNA	Long non-coding RNA
LE	laminin-type epidermal growth factor
LRR	Leucine-Rich-Repeat
maGSC	multipotent adult germline stem cell
MAPK	MAPK/ERK Kinase 1
Mbd3	Methyl-CpG-binding domain protein 3
MDM2	Mouse Double Minute 2 homolog
MEF	mouse embryonic fibroblast
MEK	MAP/ERK Kinase1
MET	mesenchymal-to-epithelial transition
miRNA	MicroRNA
MiH	minocycline hydrochloride
mTOR	mechanistic target of rapamycin
NeICD	Neogenin intracellular domain
NOD	non-obese diabetic
NRAGE	neurotrophin receptor-interacting MAGE homolog
ntESC	nuclear transferred embryonic stem cell
NURD	Nucleosome Remodeling Deacetylase
N-WASP	neuronal Wiskott-Aldrich syndrome protein
PcG	Polycomb group
PD	PD0325901
PDGF	Platelet-Derived Growth Factor
pES	parthenogenetic embryonic stem
PICK1	Protein Interacting with C kinase 1
PI3K	Phosphatidyl inositol 3 Kinase
PKA	protein kinase A
PLC γ	phospholipase γ
PRC	polycomb repressive complex
PrE	primitive endoderm
PSC	pluripotent stem cells
PKC	Protein Kinase C
RGMa	Repulsive Guidance Molecule Family a
SFK	Src family kinase

SCNT	Somatic Cell Nuclear Transfer
SHH	Sonic Hedgehog
STAT	Signal Transducers and Activators of Transcription
SWI/SNF	SWItch/Sucrose Non-Fermentable
T	Brachyury Transcription Factor
Tcf711	Transcription Factor 7 like-1
TAP	Tandem Affinity Purification
TE	trophectoderm
TF	transcription factor
TGF- β	Transforming Growth Factor β
TICs	tumor-initiating cells
TIRF	Total Internal Reflection Fluorescence
TrxG	Trithorax group
TSP-1	Thrombospondin-1
Unc	Uncoordinated
XIAP	X-linked inhibitor of apoptosis
ZU5	zona occludens 5

INTRODUCTION

CHAPTER I: PLURIPOTENCY

Pluripotent stem cells: definition and origin

Origin and type of pluripotent stem cells

Definition

Pluripotency is a transient property of epiblast cells during early embryogenesis. Pluripotency is short-lived and refers to an unrestricted developmental potential which give rise to all three embryonic germ layers –endoderm, ectoderm and mesoderm- and, eventually, to all cell types of an adult organism. Pluripotency can be captured *in-vitro* using defined culture conditions. The resulting cells are called pluripotent stem cells (PSCs.) Unlike epiblast, pluripotent cells in culture can self-renew indefinitely while retaining multilineage differentiation abilities.

Pluripotent cell types

Pluripotent stem cells are named depending on the sources of derivation or on the site from which they were obtained. Different types of PSCs include the embryonal carcinoma (EC) cells derived from teratocarcinomas, the embryonic stem cells (ESCs) derived from pre-implantation blastocyst embryos, the epiblast stem cells (EpiSCs) derived from post-implantation embryos, the parthenogenetic embryonic stem (pES) cells obtained from parthenogenetic (*in-vitro* fertilized) blastocysts (Barton et al, 1985) and multipotent adult germline stem cells (maGSCs) derived from adult testis (Guan et al, 2006).

Pluripotency may also be reinstated in cells of later developmental stages with different methods. One method includes cell culture adaptation of embryonic germ cells (Matsui et al, 1992; Resnick et al, 1992). Somatic cell nuclear transfer (SCNT) (Gurdon, 1962b; Wilmut et al, 2002), cell fusion (Blau et al, 1983) and somatic cell reprogramming with defined transcription factors (Takahashi & Yamanaka, 2006) are three approaches to reprogram somatic cells to a pluripotent state **(Figure-1)**. These artificially generated pluripotent cells exhibit molecular and functional properties similar to, and characteristic of, their embryonic counterparts.

In culture, all PSCs share similar features, such as the self-renewal and multilineage differentiation abilities. Yet, recent studies suggest that all PSCs are not equal and they may vary in their individual properties. Within the broad definition of pluripotency, different pluripotent states exist. Descriptors such as 'naïve' and 'primed' states are associated with distinguishable molecular and functional features (Hackett & Surani, 2014; Nichols & Smith, 2009; Wu & Izpisua Belmonte, 2015). (Naïve and primed states are described in 'early mouse development' section)

Discovery and derivation

The discovery of ESCs came from investigations of teratomas (benign) and teratocarcinomas (malignant). These rare tumors, found in gonads but also rarely in extra-gonadal sites, contain a variety of different cell lineages, and include well-formed structures such as teeth, bone and hair. In 1954, Stevens discovered that males of the inbred mice strain 129 sporadically developed testicular teratocarcinomas that could be maintained continuously by serial transplantation (Stevens & Little, 1954). Within ten years, the presence of tumor-initiating cells (TICs) that can develop into a teratoma or teratocarcinoma has been revealed within fetal testes (Stevens, 1964). Most importantly, it was subsequently shown

that a single cell could regenerate a multilineage teratocarcinoma indicating that such TICs harbor a very strong differentiation potential (Kleinsmith & Pierce, 1964).

In 1970s, several clonal embryonal carcinoma (EC) cell lines were derived from teratocarcinomas and these clones produced several cell types in culture (Evans, 1972; Kahan & Ephrussi, 1970; Pierce & Verney, 1961; Rosenthal et al, 1970). These cells could proliferate indefinitely, and produced teratocarcinoma following subcutaneous injection into immunocompromised mice. Furthermore, they could be differentiated into embryoid bodies, the embryo-mimicking structures that exhibit differentiation features found in early embryo, when cultured in non-adherent conditions (Martin & Evans, 1974; Martin & Evans, 1975). EC cells can carry chromosomal anomalies (Martin, 1980). Nonetheless some EC cell lines were able to colonize the host embryo and contribute to the formation of chimeras (single organism incorporating cells from different genetic lineages) when they were injected into blastocysts (Rossant & Papaioannou, 1985). This 'chimera formation assay' is described in detail below, and represents a key criterion to evaluate pluripotency. The seminal result that a subset of EC cells could respond to a host embryo environment and display functional attributes of normal embryonic cells paved indeed the way for the derivation of the first mouse embryonic stem cell (ESC) lines on a feeder cell layer (division-incompetent fibroblast). The first ESC lines were derived from inner cell masses (ICM) of early blastocyst (76 hours after detection of a plug), cultured in medium conditioned by an established teratocarcinoma stem cell line (Martin, 1981) and subsequently from growth-arrested blastocysts (Evans & Kaufman, 1981). These cells harbor a normal karyotype, give rise to teratomas when engrafted into mice and could contribute to the formation of germ-line chimeras (Bradley et al, 1984).

Mouse ESCs self-renew rapidly and continuously *in-vitro*. A key signal for sustaining their self-renewal is the cytokine Leukemia Inhibitory Factor (LIF) (Smith et al, 1988; Williams et al, 1988). (Different cell culture conditions used for derivation of

various stem cells are described in the 'early mouse developmental stages' section.) Although ESCs and EC cells share similarities such as teratoma formation and chimera formation abilities, the fact that EC cells could be derived only via formation of tumors and were genetically abnormal, called their overall similarity to embryo cells in question.

Primed human ES cell derivation was achieved several years later in the presence of activin and FGF2 rather than LIF (Thomson et al, 1998). Within a decade, by using same culture conditions for human ESCs, another type of mouse pluripotent stem cells: epiblast stem cells (mEpiSCs), were derived from post-implantation epiblast of murine embryos (Brons et al, 2007; Tesar et al, 2007). The unique qualities of these stem cells are correlated with their derivation from different stage blastocysts. The mEpiSCs differs from mESCs in morphology, transcription factor network, signaling, methylation levels and functionalities. For example, naive mESCs contribute to chimera formation when injected into a blastocyst, whereas primed mEpiSCs efficiently engraft into mouse gastrula-stage embryos, but not vice versa (Huang et al, 2012; Wu et al, 2015). These findings indicate that mEpiSCs are distinct from mESCs, but closer to primed human ESCs. Thus, mEpiSCs represent a model to distinguish mouse and human ESCs.

Reprogramming methods and established pluripotent cell types

Aside from *in-vivo*-derived stem cell types, it is possible to establish pluripotent cells that could give rise to an entire organism from terminally differentiated cells due to cellular plasticity. Three distinct experimental approaches to reprogram to a pluripotent state: nuclear transfer, cell fusion and transcription factor-transduction are depicted below (**Figure-1**).

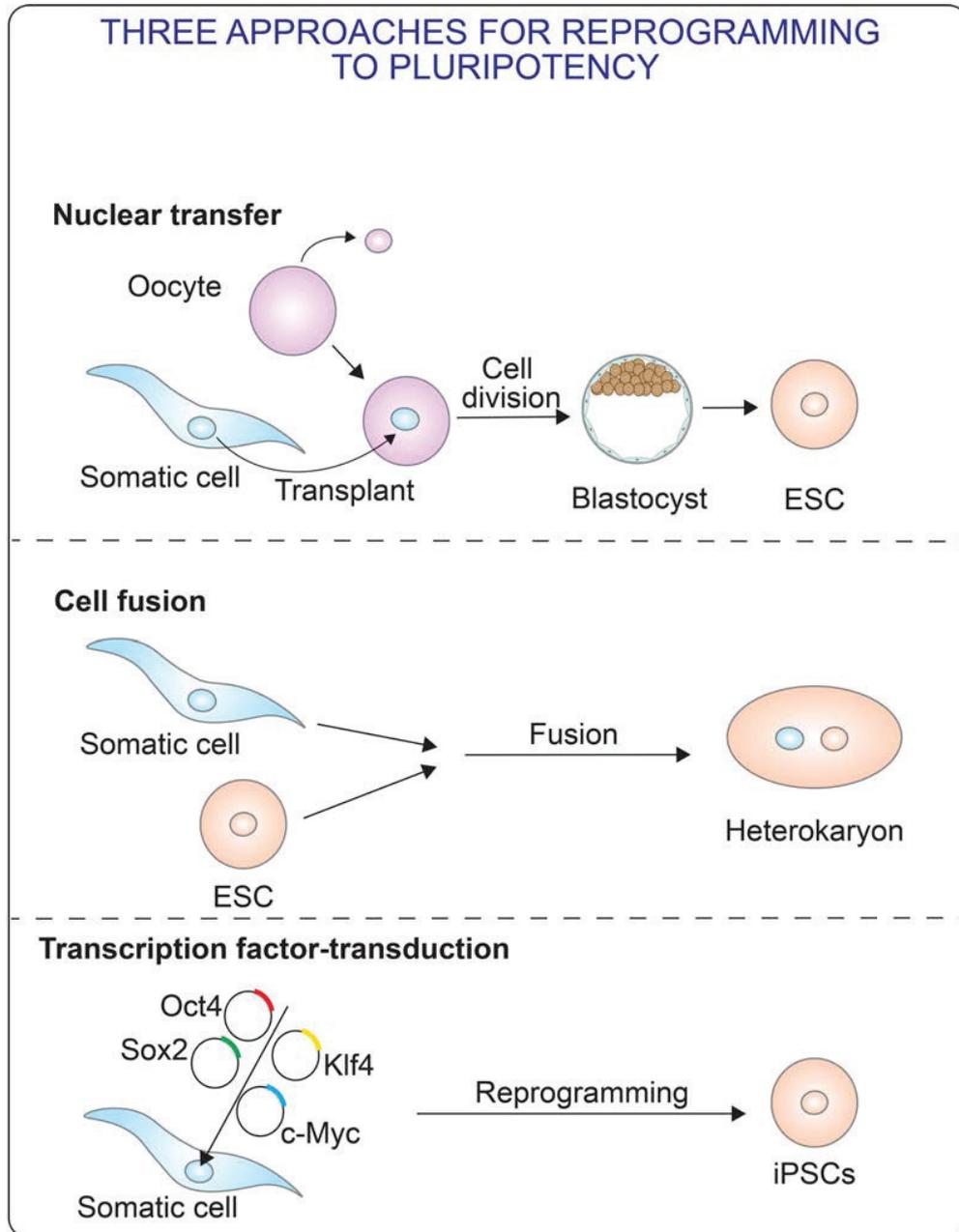


Figure-1: Three approaches for reprogramming to pluripotency.

During nuclear transfer, the nucleus of a somatic cell is transplanted into an enucleated oocyte. A blastocyst is developed from this oocyte. mESCs are derived from this blastocyst. In cell fusion approach, two distinct cell types are combined to form a single entity. The resultant fused cells can be heterokaryons or hybrids. The transcription factor-transduction method can be used to form induced pluripotent stem cells (iPSCs), which have similar properties to ESCs and can be generated from almost any cell types in the body via the introduction of four genes (Oct4, Sox2, Klf4 and c-Myc). The pluripotent state is heritably maintained, and vast numbers of cells may be generated, making this approach advantageous for clinical applications. Adapted from (Yamanaka & Blau, 2010).

Somatic cell nuclear transfer (SCNT)

The first reprogramming method is Somatic Cell Nuclear Transfer (SCNT), or cloning. Following cloning, the reprogrammed cells are called nuclear transferred embryonic stem cells (ntESCs). Elegant cloning studies in frogs showed for the first time that the transfer of nuclei from early blastocysts into enucleated oocytes resulted in colonies of swimming tadpoles (Briggs & King, 1952). With the same experimental conditions, but using the *X.laevis* species, Gurdon succeeded in obtaining a viable adult organism (Gurdon, 1962a; Gurdon, 1962b). This demonstrated that the nuclei of somatic cells maintain all required genetic information, and that by experimental manipulation they can be reprogrammed to an embryonic pluripotent state, that is, rejuvenated. However, it took 36 years to clone the first mouse (Wakayama et al, 1998). In human zygotes, SCNT is accepted as unsuccessful because of developmental arrest which occurs before blastocyst stage (Egli et al, 2011). However, the optimized SCNT conditions paved the way for the derivation of human ntESCs that share similar gene expression and differentiation profiles with embryo-derived ESCs (Tachibana et al, 2013).

Cell Fusion

Another reprogramming method, cell fusion, involves fusing pluripotent ESCs and differentiated cells to generate a pluripotent heterokaryon, which is a short-lived and non-dividing multinucleate fusion product of two distinct cell types. The three ways to generate heterokaryons are: electrical cell fusion, polyethylene glycol cell fusion and sendai virus-induced cell fusion. Cell fusion was first documented in 1983, with experiments showing that silenced muscle-specific genes in human amniocytes are activated after cell fusion with mouse muscle cells (Blau et al, 1983). In the same year, it was demonstrated that inactivated X chromosomes in female somatic cells, such as thymocytes or bone marrow cells, could be reactivated by fusion with teratocarcinoma-derived cells in which both X chromosomes were

active (Takagi et al, 1983). Mouse hybrid cells were established by fusing adult thymocytes with ESCs and were able to contribute to all germ layers of chimeric embryos (Tada et al, 2001). Human somatic hybrid cells were produced by fusing human fibroblasts with a human ESC line (Cowan et al, 2005). These findings demonstrated that reprogramming factors in embryonic stem cells are able to delete or reset the memories of somatic cells. However, to use these hybrid ES-like cells for clinical applications, the elimination of the ES cell chromosomes either before or after cell fusion is required. The selective deletion of chromosomes is possible but impractical for the complete set of chromosomes (Matsumura et al, 2007).

Somatic cell reprogramming via transcription factor-transduction

In 2006 Takahashi and Yamanaka made a groundbreaking discovery. They found that differentiated mouse and human somatic cells could be reprogrammed to a pluripotent state by a set of defined transcription factors (Takahashi et al, 2007; Takahashi & Yamanaka, 2006). They called these cells induced pluripotent stem cells (iPSCs). One of the most critical findings in the generation of induced pluripotent stem cells (iPSCs) was the identification of the key pluripotency factors through the establishment of the candidate genes list. To do so, researchers used *in silico* subtraction to identify cDNAs that are specifically enriched in ESCs compared to somatic cells, and then obtained cDNAs encoding novel genes that were designated ES cell-associated transcripts (ECATs) (Mitsui et al, 2003). The generation of knockout mice and ESCs with the disruption of one of the ECATs, (F-box only protein 15, Fbxo15), was critical in the discovery of iPSCs (Tokuzawa et al, 2003). In this case, the Fbxo15 gene was disrupted by the β -galactosidase and neomycin resistance cassette (β -geo), and its expression was subsequently used as a reporter of *Fbxo15* promoter activity. As expected, with no expression of Fbxo15 in somatic cells, the mouse embryonic fibroblasts (MEFs) were not resistant to neomycin treatment, however Fbxo15- β -geo ESCs, due to strong Fbxo15 promoter activity, could survive even in the presence of high levels of neomycin. These MEFs

were used as a system to screen for reprogramming factors based on transducing various combinations of candidate genes composed of 24 factors and evaluating the ability of MEFs to survive neomycin treatment. By iteratively adding fewer factors in the candidate cocktail with each transduction, they were able to identify four transcription factors, Oct4, Sox2, Klf-4 and c-Myc (OSKM) that were sufficient to reprogram somatic cells. These factors are called as Yamanaka factors.

These results were notable because, among the different reprogramming methods, OSKM-mediated-reprogramming is the easiest to perform and control. Nonetheless, the process is inefficient. Therefore, identifying roadblocks hindering somatic cell reprogramming to pluripotency is crucial for the creation of high-quality pluripotent cells and may be useful for therapeutic applications. Parts of these thesis studies focus on identifying new factors that could modulate the iPSCs generation process. In the next chapter, I briefly describe different phases of reprogramming, the limiting and potentiating reprogramming factors and potential applications of reprogramming.

Considerations for somatic cell reprogramming

Phases of OSKM-mediated-reprogramming and resulting cell fates

Buganim and colleagues showed that the reprogramming process may broadly be divided into two phases: stochastic phase and deterministic phase (Buganim et al, 2012; Buganim et al, 2013). In their study, they performed single cell analysis to examine the molecular events associated with the mechanism of action for the Yamanaka factors. Using arrays, they analyzed the expression of 48 genes including those for ESC chromatin remodeling, cell cycle regulation, signal transduction, and pluripotent markers in single cells sorted from different stages of OSKM-induced reprogramming representing early, intermediate, and fully reprogrammed iPSCs.

They concluded that reprogramming starts with a long stochastic phase of gene activation, and continues with a shorter, hierarchical, more deterministic phase of gene activation. OSKM induction initiates stochastic gene expression, which leads to various outcomes such as apoptosis, senescence, transformation, transdifferentiation and the desired outcome; reprogramming to pluripotency. The undesired products hinder the process, with particularly significant contributions being apoptosis and senescence. In the early phase, reprogrammable cells increase proliferation, undergo changes in histone modifications at somatic genes, initiate mesenchymal-to-epithelial transition (MET) and activate DNA repair and RNA processing (Buganim et al, 2013). Reprogramming is followed by an intermediate phase in which cells undergo a stochastic activation of pluripotency markers (Buganim et al, 2012) and a transient activation of developmental regulators (Polo et al, 2012). In the deterministic late phase, cells stabilize the pluripotent state. The retroviral transgenes are silenced, the cytoskeleton is remodeled to an embryonic stem cell-like state, the epigenome is reset and so that the endogenous core pluripotency circuitry (the core pluripotency transcription factors are mentioned in 'molecular regulation of pluripotency' section) is activated (Buganim et al, 2012; Golipour et al, 2012; Polo et al, 2012). In this model, probabilistic events decrease and hierarchical events increase as the cell progresses from a fibroblast to an iPSC. This model helps better understanding the molecular mechanisms of iPSC generation process and highlights the reasons of its inefficacy.

Improving iPSCs reprogramming

To date, several biological processes are documented as roadblocks or modulators of reprogramming. These include epigenetics barriers (histone marks, chromatin modifiers) (Buganim et al, 2013; Rais et al, 2013), cell death and senescence (p53, Ink4/Arf) (Li et al, 2009; Marion et al, 2009; Rais et al, 2013; Utikal et al, 2009), ubiquitination of key substrates, and modulation of endocytosis or cell adhesion and motility pathways (Qin et al, 2014). By contrast, several factors identified to enhance reprogramming or to substitute for reprogramming factors include small

molecules, RNA-based regulators, growth factors, vitamin C, cytokines and three-dimensional microenvironments (Bar-Nur et al, 2014; Caiazzo et al, 2016; Esteban et al, 2010; Hou et al, 2013; Judson et al, 2009; Qin et al, 2014; Sandmaier & Telugu, 2015; Silva et al, 2008). These recent findings suggest mechanisms to optimize the efficiency of iPSCs generation, and therefore may have important medical applications.

Another limiting issue regarding the clinical applications of iPSCs is the use of retroviral vectors bearing OSKM, which integrate into to the genome of host cells. These vectors may cause disruption or aberrant activation of neighbor genes and pose a risk of later reactivation of the reprogramming factors themselves. For example, the reactivation of c-Myc, a known oncogene, sporadically induces tumor formation in iPSC-derived chimeric mice (Okita et al, 2007). This result suggests that retroviral induction of reprogramming factors has to be avoided for clinical application. Several integration-free methods have been developed to ensure the transient expression of the reprogramming factors. These include adenoviruses (Stadtfield et al, 2008), plasmids (Okita et al, 2008; Yu et al, 2009), piggyBac transposon (Kaji et al, 2009) , Sendai viruses (Fusaki et al, 2009), synthetic mRNAs (Warren et al, 2010), recombinant proteins (Kim et al, 2009) , chemical compounds (Kim et al, 2009) or combinations of chemically defined media and recombinant matrix proteins (Chen et al, 2011; Nakagawa et al, 2014). Such techniques are becoming the preferred approaches for the generation and maintenance of iPSCs.

Potential applications of iPSCs reprogramming

Together these advancements have been crucial to enable the use of iPSCs, and iPSCs-derived cells for medical purposes in non-human organisms. In preclinical studies, iPSCs have been used in rats for the treatment of Parkinson's disease (Wernig et al, 2008), and in mice and non-human primates for treatment of spinal cord injury (Kobayashi et al, 2012; Tsuji et al, 2010). Moreover, iPSCs are also used

to generate disease models and for chimeric organ complementation, which is the generation of human organs in animal hosts via interspecies chimeric complementation (Shi et al, 2017; Wu et al, 2016). Furthermore, drug screening can be carried out *in vitro* using these iPSC-based disease models to determine whether therapeutic drug candidates ameliorate or correct aberrant pathways. The iPSC approach is considered particularly advantageous for therapeutic applications because using patient-derived iPSCs overcomes the ethical issues associated with the use of embryonic tissue, as well as the critical problem of immunological rejection. In 2014, the first transplantation of iPSC derived-cells was conducted in Japan. In this case, iPSC-derived retinal pigment epithelium (RPE) cells were used to attempt to treat a patient with 'Wet' Age-Related-Macular degeneration (AMD) (Hotta & Yamanaka, 2015). This disease may cause vision loss due to abnormal blood vessel growth. The Wet form of AMD is the most aggressive form of the disease, and while anti-angiogenic agents can slow disease, there are no cures yet. These past studies collectively suggest that a better understanding of the molecular mechanisms of reprogramming, the discovery of additional enhancers of reprogramming and the development of new technologies enabling potential iPSC-based medical approaches will be useful, even essential, to advance basic and translational research.

Assessment of pluripotency features

A number of assays have been developed to define ESC self-renewal and pluripotency. These *in-vitro* and *in-vivo* assays permit the identification and evaluation of novel putative regulators of ESC identity.

***In-vitro* assays**

Colony formation

Among *in-vitro* assays, colony formation assay (CFU, colony-forming-unit) is used to test self-renewal ability of pluripotent stem cells. mESCs have the capacity to self-renew at clonal density, allowing a quantitative measurement of the self-renewal capacity of individual cells. In a colony formation assay, single cells are seeded at a very low cell density (around 50 cells per cm²) and allowed to proliferate for five to six days. The colonies obtained are then scored for a marker of undifferentiated ES cells, such as alkaline phosphatase or a transgene reporter. The total and the relative numbers of undifferentiated, mixed, or fully differentiated colonies reflect the self-renewal efficiency. The assay can be adapted to test stability of the undifferentiated state. In this type of 'commitment assay', cells are plated at low density then cultured for a period without self-renewal input such as the cytokines LIF and Bone Morphogenic Protein (BMP). The number of undifferentiated colonies formed upon re-addition of cytokines indicates the persistence of self-renewal capacity. This assay has been used successfully to screen for and characterize genes that mediate exit from pluripotency and entry into differentiation (Betschinger et al, 2013; Karwacki-Neisius et al, 2013).

Embryoid body formation and other *in-vitro* differentiation assays

The *in-vitro* embryoid body (EB) formation assay is used to test the differentiation potential of PSCs into the derivatives of the three embryonic germ layers (ectoderm, mesoderm and endoderm). The cells are seeded in non-adherent dishes (or in hanging-drops of media) and cultured without self-renewal input (eg, LIF). The cells interact, assemble, and form spheroids that are subsequently enveloped by a layer of primitive endoderm cells (Capo-Chichi et al, 2005). Lineage markers expression

could be detected during differentiation (generally from 2 days to 10 days after for mouse PSCs). *In-vitro* differentiation can be tested using cocktails of differentiation inducing cytokines, morphogens or chemicals, which direct the cells towards a defined lineage. However, to test the full lineage repertoire and functionality of differentiation, *in-vivo* assays are required.

***In-vivo* assays**

Teratoma formation

Among *in-vivo* assays, the teratoma formation assay assesses the spontaneous generation of differentiated tissues from the three germ layers following the injection of cells into immune-compromised mice. There are four main injection sites for teratoma: (1) intramuscular, (2) subcutaneous, (3) in the testis, and (4) under the kidney capsule. Histologic analysis of teratomas is not quantitative. Moreover, incompletely reprogrammed cells can generate masses that superficially resemble teratomas potentially leading to misinterpretation (Chan et al, 2009). Since teratomas are not generated from single cells, the teratoma assay assesses developmental potency at a population-based level. These considerations indicate that teratoma formation is not the most stringent assay to evaluate pluripotency.

Chimera formation

Another *in-vivo* assay, the blastocyst chimera formation, measures whether cells can contribute to development when introduced into host embryos by injection into blastocysts (Nagy et al, 1990). High-quality pluripotent cells support normal development and generate high-grade chimaeras with extensive colonization of all embryonic tissues including the germ line. Cell quality is a critical determinant for the assay, as low quality pluripotent cells produce reduced levels of chimerism and may also exhibit reduced embryo viability.

Germline transmission

The germline transmission assay is an extension of the chimera assay and involves breeding chimeric mice to produce all-donor PSC-derived offspring. This assay thus demonstrates the capacity of the cells to generate functional gametes. This assay is a robust indicator of chromosomal integrity and of functional pluripotency.

Tetraploid complementation

The tetraploid complementation assay measures the capacity of mouse pluripotent cells to direct development of an entire organism. Donor PSCs are introduced into tetraploid (4n) host blastocysts, as generated by electrofusion of blastomeres at the two-cell stage. The 4n blastocysts are not able to sustain normal embryonic development beyond mid-gestation, and thus the resulting embryos are derived only from donor pluripotent cells (2n), while the extra-embryonic tissues develop from tetraploid cells and support the donor cells (Nagy et al, 1993).

Single cell chimera

Another very stringent assay consists in the injection of a single-donor mouse pluripotent cell into a morula or blastocyst (Wang & Jaenisch, 2004) . Although both the single-cell chimerism and tetraploid complementation assays exhibit high failure rates, they are considered to be among the most definitive ways of demonstrating pluripotency.

Interspecies chimera

Interspecies chimerism, the formation of a chimera using a different species than the source PSCs, is not held to be a standard assay. However, it is currently

becoming a strategy to compare distinct pluripotent states (naïve, intermediate or primed) (Wu et al, 2015). Recently, scientists demonstrated that naïve hPSCs robustly engraft in both pig and cattle pre-implantation blastocysts but show limited contribution to post-implantation pig embryos. Instead, an intermediate hPSC type exhibits higher degree of chimerism and is able to generate differentiated progenies in post-implantation pig embryos (Wu et al, 2017). Accordingly, interspecies chimerism has become the subject of interest for humanized mouse models of disease and for human organ generation in species close to human such as cattle and pig.

Early embryonic development

Early mouse developmental stages

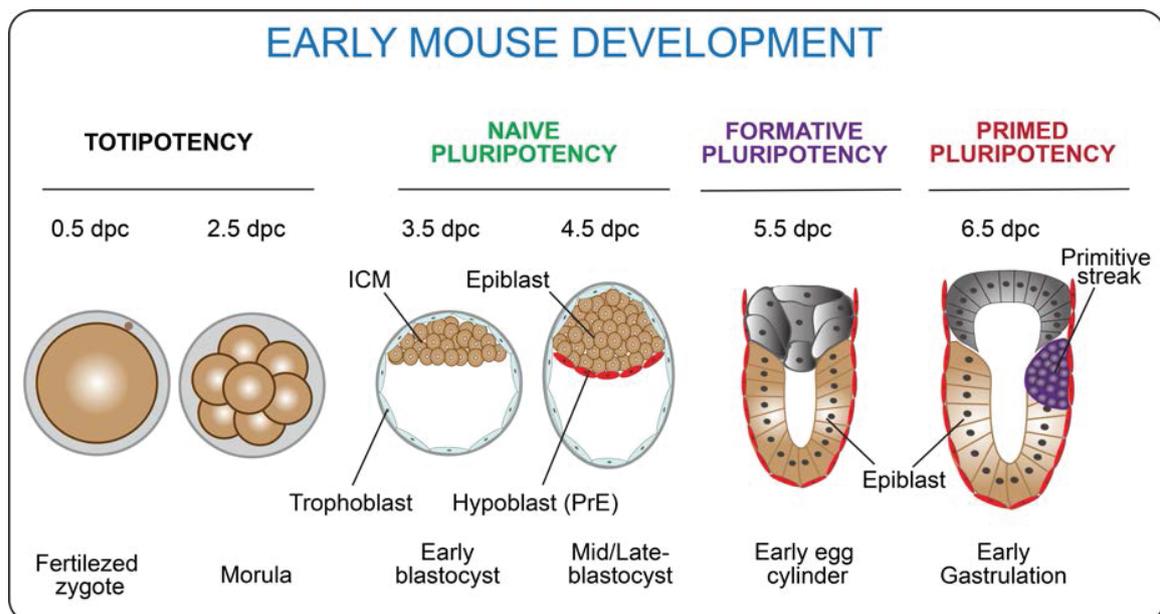


Figure-2: Overview of early mouse development

Totipotency starts upon fertilization and persists until early blastocyst stage. Naive pluripotency is established at the blastocyst stage and persists until implantation. Upon implantation, naïve pluripotency give place to formative and primed pluripotency. The terms totipotency and pluripotency apply to the embryonic lineage (light brown) but not to the hypoblast (red), trophoblast (grey), extra-embryonic ectoderm that is arising from trophoblast (grey), primitive streak (purple)

Totipotency

From “Fertilized Zygote” to “Polarized Embryos”

Totipotency is the capacity to produce all embryonic and extraembryonic lineages within the fertilized egg. Upon fertilization, cells generated by cleavage divisions are referred to as blastomeres. The cellular compaction is the first event of totipotency and occurs at the late 8-cell stage (2.75 days post coitum (dpc)). The cellular compaction initiates establishment of the future compartments. The outer cells transition towards the first extraembryonic lineage, the trophectoderm, which is required for implantation and will give rise to the placenta (Rossant & Vihj, 1980). By contrast, the inner cells form the inner cell mass (ICM) of the early blastocyst by maintaining expression of the transcription factor Oct4, which is downregulated in outer cells. In the absence of Oct4, the inner cells differentiate into trophectoderm (Nichols et al, 1998). During compaction, E-cadherin regulates the adhesion of the inner cells (Johnson et al, 1986). The outer cells become polarized by asymmetric localization of polarity proteins such as aPKC, Par3 and the cytoskeletal adaptor protein ezrin (Johnson et al, 1986; Louvet et al, 1996; Plusa et al, 2005). Moreover, Hippo pathway signaling is specifically active within the inside cells (Nishioka et al, 2009). Additionally, cellular adhesion factors (Johnson et al, 1986) are important regulators of early inner cell establishment during totipotency. The inner cells isolated from 3.5 dpc embryo lose their capacity to form trophectoderm (Rossant & Lis, 1979). Subsequently, the inner cells (inner cell mass, ICM) segregate into pluripotent epiblast and hypoblast (also known as primitive endoderm (PrE)) (Chazaud et al, 2006; Gardner & Rossant, 1979). **(Figure-2 and -3)**

Pluripotency

Pluripotency is a transient state *in-vivo* that occurs during early embryogenesis. Different stages of pluripotency is described below and depicted in Figure-3 (Figure-3).

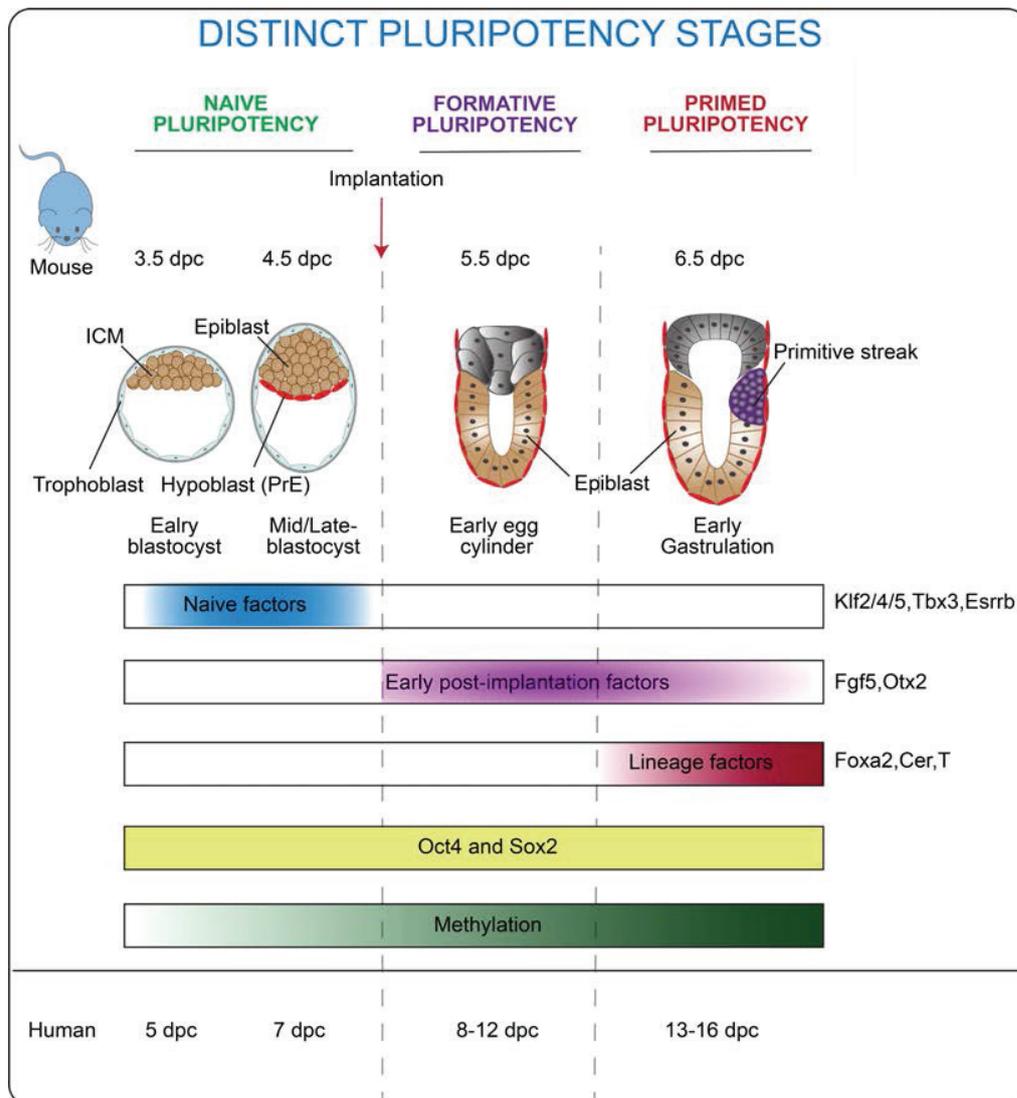


Figure-3: Progression from naïve to primed pluripotency and corresponding conceptual pluripotent stages.

The mature blastocyst comprises three cell lineages: epiblast (light brown), hypoblast (or primitive endoderm) (red) and trophoblast (grey). By E6.5 lineage priming has commenced. Naïve ESCs are derived from naïve epiblast and primed ESCs are derived from primed epiblast. Naïve factors are expressed exclusively by naïve epiblast and naïve ESCs. Early post-implantation epiblast (the intermediate formative stage) expresses early post-implantation factors, which exhibit a decrease in expression levels upon gastrulation. Primed epiblast expresses lineage factors. Oct4 and Sox2 are expressed during all periods of pluripotency. DNA methylation increases during pluripotency progression. Pluripotency progression in human passes through same stages with mice but persist longer than mice.

Naïve pluripotency in-vivo and in-vitro

Naïve pluripotency emerges just after segregation at 3.5 dpc embryos and continues until implantation at 4.75 dpc. The epiblast is the pluripotent embryonic compartment and PrE contributes to yolk sac development (Chazaud et al, 2006). In 3.5 dpc embryos, epiblast and PrE transcription factors, Nanog and Gata6, are expressed in the ICM in a random manner, dependent on Mitogen-Activated Protein Kinase (MAPK) signaling, which is described in detail below. Segregation occurs at 4.5 dpc (Chazaud et al, 2006). Fibroblast Growth Factor 4 (FGF4) is required for, and drives, epiblast and PrE segregation (Ohnishi et al, 2014). In mouse, FGF/ Extracellular Regulated Kinase (ERK) signaling is the main driver of pluripotent epiblast and PrE segregation process (Arman et al, 1998; Rappolee et al, 1994). A difference in FGF responsiveness leads the activation of different transcriptional programs in epiblast and PrE (Guo et al, 2010; Ohnishi et al, 2014). Epiblast express pluripotency markers such as Nanog and Sox2, PrE cells express lineage markers such as Gata6 and Sox17 and FGF receptors FGFR2, FGFR3 and FGFR4 (Chazaud et al, 2006; Guo et al, 2010; Niakan et al, 2010). All cells, by contrast, express FGFR1 (Ohnishi et al, 2014). In agreement with these *in vivo* observations, modulation of FGF signaling in culture influences ICM cell fate. Inhibition of FGF/ERK signaling directs ICM to become epiblast, while the addition of exogenous FGF4 and FGF2 biases cell identity toward PrE (Nichols et al, 2009b; Yamanaka et al, 2010).

Naïve pluripotency is not restricted to epiblast and can be captured *in-vitro* as ESCs (Evans & Kaufman, 1981; Martin, 1981). Pluripotent ESCs acquire the ability to self-renew indefinitely, thereby producing an unlimited quantity of cells while retaining their pluripotent status *in-vitro*. Mouse ESCs can be derived from ICMs of 3.5 dpc and 4.5 dpc embryos very efficiently, and from 2.5 dpc embryos, though with very low efficacy (Boroviak et al, 2014). Mouse ESCs are considered to be in the naïve pluripotent state because they display a molecular signature reminiscent of naïve

epiblast of a mature 4.5 dpc blastocyst (Boroviak et al, 2014), and have the ability to form chimeras following blastocyst injection. Yet according to another hierarchical clustering, mESC transcriptome more closely resembles the 5.5dpc epiblast (Bulut-Karslioglu et al, 2016). The differences in the results and conclusions may be significant, but could also be due to the different serum composition in the cell culture media and/or representative of the specific gene set chosen for clustering.

Mouse ESCs are sourced from pre-implantation epiblast of the ICM and can be cultured in growth medium containing leukemia inhibitory factor (LIF) and serum, referred as conventional culture conditions (Smith et al, 1988). The cells cultured in conventional culture conditions reside in a metastable state exhibiting heterogeneity in morphology with fluctuating pluripotency factors expression, weak expression of lineage markers, and a high rate of spontaneous differentiation owing to ERK signaling (Chambers et al, 2007; Toyooka et al, 2008; Wray et al, 2010; Ying et al, 2008). The requirement for serum can be replaced by BMP4 (Ying et al, 2003). Mouse ESCs can be derived and be cultured without serum or LIF, by combining two inhibitors (2i) of both MEK and GSK3 pathways (Ying et al, 2008). Under these conditions, the MEK inhibitor limits differentiation, while the GSK3 inhibitor promotes self-renewal together with LIF. The embryonic stem cells that are cultured in 2i conditions are considered to be “ground state” naïve mESCs (Ying et al, 2008). The use of inhibitors increases the efficiency of ESC derivation from 4.5dpc blastocyst (Nichols et al, 2009b). This is true even for strains of mice that are otherwise non-permissive, notably including non-obese diabetic (NOD) mice from which ESCs cannot be derived using standard culture methods (Kiyonari et al, 2010; Nichols et al, 2009a). The general approach is robust, as similar observations have been made using non-permissive strains of rat (Buehr et al, 2008; Li et al, 2008).

The transcriptomic and epigenomic features of ground state mESCs seem to reflect an earlier developmental stage, as early as the inner cell mass of early embryos (3.5 dpc) or the 2.5 dpc morula (Bulut-Karslioglu et al, 2016). Cells cultured in ground state conditions exhibit known hallmarks of naive pluripotency including global

DNA hypomethylation, lack of bivalent marks on chromatin (described in 'epigenetics of pluripotency regulators') and homogenous expression of naïve pluripotency genes such as *Esrrb* (Marks et al, 2012).

The 'ground state' has been widely used in stem cell research as a model to reflect naïve pluripotency. This state promoted our understanding of ESCs biology and facilitated the capture of naïve ESCs *in-vitro*. Moreover, the utilization of small molecules is very promising for translational applications for medical purpose. In this context, it is important to establish whether the sustained propagation of naïve ESCs in ground state culture conditions affects their stability and functionality. Recently, it has been suggested that although short-term inhibition of ERK signaling in ESCs helps to maintain an ICM-like epigenetic state, the prolonged culture of mESCs in ground state conditions (2i and LIF) results in irreversible epigenetic and genomic changes that impair their developmental potential. This occurs due to the inhibition of ERK signaling and subsequent downregulation of DNA methyltransferases and their cofactors (Choi et al, 2017a). This issue is overcome by the replacement of the ERK inhibitor with a SRC inhibitor. It seems that SRC inhibition preserves the epigenetic and genomic integrity, as well as the developmental potential of ESCs, though the reasons this should be so are not completely clear.

Primed pluripotency *in-vivo* and *in-vitro*

Exit from naïve pluripotency starts upon implantation around 4.75 to 5.75 dpc mouse embryos and continues till gastrulation around 7 dpc of mouse embryogenesis (**Figure-3**). During implantation epiblast cells become a highly polarized rosette. This transformation requires basal membrane-stimulated integrin signaling that coordinates polarization of epiblast cells and their apical constriction, a prerequisite for lumenogenesis (Bedzhov & Zernicka-Goetz, 2014). Thus, the extracellular matrix (ECM) triggers the self-organization of the embryo's stem cells.

mEpiSCs can be derived from a broad range of post-implantation epiblast tissues (E5.5 to E8 day) using basal medium supplemented with activin, FGF2 with optional addition of KSR (knockout serum replacement) and feeders (Brons et al, 2007; Kojima et al, 2014; Tesar et al, 2007). mEpiSCs show features distinct from mESCs and are in a more advanced pluripotent state developmentally; that is 'primed' pluripotent state. Primed EpiSCs express Oct4 and Sox2, but do not express naive pluripotency factors except for Nanog. However, they express the early postimplantation epiblast marker FGF5, and lineage-specific factors, such as Brachyury Transcription Factor (T), Forkhead Box A2 (Foxa2) and Cerberus (Cer) in a highly heterogeneous manner (Kojima et al, 2014).

mEpiSCs have been shown to retain functional properties of the post-implantation epiblast, in that they can be differentiated to somatic lineages *in-vitro* and *in-vivo* by teratoma formation (Kojima et al, 2014). Primed EpiSCs are not efficient at generating blastocyst germline chimeras when they are injected into pre-implantation epiblast. Nonetheless, they can broadly engraft into the post-implantation epiblast and form *ex vivo* epiblast chimeric embryos because they have altered molecular characteristics and correspond to a more advanced developmental stage in comparison to the host pre-implantation environment (Huang et al, 2012; Kojima et al, 2014; Wu et al, 2015). EpiSCs can, however, form low-contribution chimeric embryos when injected into host post-implantation embryos *ex vivo* (Huang et al, 2012). ERK signaling is one of the key drivers initiating exit from naïve pluripotency and entry into primed pluripotency and to lineage commitment (Arman et al, 1998; Kunath et al, 2007). Primed mEpiSCs can be also generated from naïve mESCs in the continuous presence of activin and FGF2 (Guo et al, 2009). Moreover, primed mEpiSCs can be reprogrammed to a naïve pluripotent state by exogenous expression of naïve transcription factors such as Klf4, Nanog or Esrrb (mentioned in ground state & naïve epiblast transcription factors) (Festuccia et al, 2012; Guo et al, 2009; Silva et al, 2009). These results indicate the cellular plasticity between different pluripotent states.

Pluripotency in human

Human and mouse early development share similarities, exhibiting distinct pluripotency states. However, the duration of pluripotency is different between the two species. Pluripotency emerges within ICM of the blastocyst and persists until somitogenesis. This period lasts 4-5 days in mouse and approximately two weeks in human embryos (**Figure-3**).

The derivation of ESCs from human blastocysts (hESCs) was first performed in media containing FGF2 and activin on feeder cells (Thomson et al, 1998). Later studies also allowed derivation of feeder-independent hESCs in defined media (Ludwig et al, 2006). Despite similar embryonic origins, there are several additional noticeable differences between hESCs and mESCs. For example, the colony morphology of mESCs is “dome” shaped, while hESCs appear flattened. There are differences in some pluripotent markers differ between human and mouse ESCs such as the absence of SSEA-1 expression in hESCs. Importantly and unlike mESCs, hESCs are sensitive to single- cell dissociation and thus need to be passaged as small clumps. Signaling pathways involved in the maintenance of the human and mouse ESC pluripotency programs are also different: instead of LIF and BMP4, hESCs are dependent on FGF and activin for their maintenance in an undifferentiated state (Vallier et al, 2005).

However, hESCs exhibit features resembling the characteristics of mouse EpiSCs including colony morphology, low single-cell cloning efficiency, and signaling dependency. Both hESCs and mouse EpiSCs could also be obtained directly from pre-implantation blastocysts (Najm et al, 2011). The similarities shared between hESCs and mouse EpiSCs suggest that, during derivation, isolated human ICM likely continue on their developmental trajectory in culture to a developmentally more advanced state and acquire an EpiSC-like identity. Like mouse EpiSCs, conventional hESCs are also classified as primed PSCs. However, hESCs exhibit molecular

signatures distinct from mouse EpiSCs (Chia et al, 2010). This may suggest that different types of primed pluripotent states exist between different species.

A naïve state in hPSCs was reported in 2010, which relied on continued transgene expression and could be propagated in mouse ground state culture (2i+LIF) (Hanna et al, 2010). However, these cells could not be maintained long-term independent of the transgene expression, suggesting that the achieved state was not a 'true' stable naïve state. Several recent reports identified the transgene-free naïve-like hPSCs, although they show molecular differences between each other (Gafni et al, 2013; Ware et al, 2014).

Other distinct pluripotency states in mouse and human

Aside from naïve and pluripotent stem cell states, the existence of a transitional, intermediate or formative state of pluripotency has also been identified (Kalkan & Smith, 2014; Wu & Izpisua Belmonte, 2015) (**Figure-3**). These transitional cells with similarities in gene expression profile to early post-implantation epiblast (E5.5) have been identified during the differentiation of ESCs. These post-implantation epiblast-like cells (EpiLCs) are transient and form from naïve ESCs at around two days after withdrawal of 2i and LIF, following the addition of EpiSC medium containing KSR (Buecker et al, 2014; Hayashi et al, 2011). EpiLCs do not exhibit ground state naïve pluripotency markers such as *Esrrb* or *Klf2*, but express early post-implantation epiblast markers such as *FGF5*, *Otx2* along with *Oct4* and *Sox2*. Transcriptome profiling places them close to the early post-implantation epiblast and distinguishes them from EpiSCs which express later lineage markers such as *Foxa2*, *Cer* and *T*. Consistent with this identity, and unlike EpiSCs, EpiLCs can be differentiated efficiently to the primordial germ cells and give rise to functional gametes, a property of the pre-gastrulation intermediate epiblast (Hayashi et al, 2012; Hayashi et al, 2011). Recently, an intermediate hPSC type was identified as well. These cells exhibit higher degree of chimerism and enable

generation of the differentiated progenies in post-implantation pig embryos (Wu et al, 2017). The discovery is very promising for human organ generation in other species.

In addition to this intermediate state, a totipotent-like state with the potential to contribute to both embryonic and extra-embryonic lineages has been described (Macfarlan et al, 2012). Macfarlan et al. discovered a rare transient population of mESCs with embryonic two-cell (2C)-like features that express MERV-L retrotransposons. These 2C-like cells lack some key pluripotency proteins, including Oct4, Sox2, and Nanog. Surprisingly, they acquired a developmental potential reminiscent of totipotency. Remarkably, nearly all ESCs cycle in and out of this totipotent-like state via a process partially controlled by histone-modifying enzymes, which are described below. Most recently, however, an in-depth single-cell RNA-sequencing analysis showed that 2C-like cells are globally more similar to blastocysts than to two-cell-stage embryonic cells (Kolodziejczyk et al, 2015). Thus, the true identity of 2C-like cells remains unclear. A recent study demonstrated that regulating the levels of miR-34a could produce bi-potential cells from murine pluripotent stem cell cultures, with a high efficiency. They revealed that miR-34a^{-/-} PSCs have an extended cell-fate potential and can generate both embryonic and extra-embryonic lineages (Choi et al, 2017b). Recently, Yang et al. identified a chemical cocktail enabling the derivation of stem cells with unique functional and molecular features from mice and humans. These cells are called as extended pluripotent stem (EPS) cells. Mouse EPS cells are capable of chimerizing both embryonic and extraembryonic tissues with totipotency features. Furthermore, human EPS cells exhibit interspecies chimeric competency in mice (Yang et al, 2017).

Altogether these collective works provide evidence that different pluripotent states exist *in-vivo*, which can be captured *in-vitro*. The features and cell culture conditions of human and mouse totipotent-like and the different pluripotent states are listed in Table-1 (**Table-1**). The cellular plasticity allows transitions between different

states. Therefore, a better understanding of the molecular mechanisms enabling such transitions between dynamic and distinct pluripotency states is critical to accelerate the use of PSCs in regenerative medicine-based approaches.

Examples of the Different 'States' of Mouse and Human PSCs and Their Properties				
Species	State	Culture Conditions	References	Features
Mouse	totipotent- like	LIF/Serum	(Macfarlan et al, 2012) (2C-like)	embryonic and extra-embryonic lineage contributions
		2i/LIF	(Morgani et al, 2013) (Hex+ ESCs)	embryonic and extra-embryonic lineage contributions
		LIF/Serum	(Choi et al, 2017b) (mir-34a ^{-/-} ESCs and iPSCs)	embryonic and extra-embryonic lineage contributions; chimeric competency
		LCDM	(Yang et al, 2017) (EPS cells)	embryonic and extra-embryonic lineage contributions; chimeric competency at single cell level
	naive	Serum; LIF/BMP4	(Evans & Kaufman, 1981; Martin, 1981; Smith et al, 1988; Ying et al, 2003) conventional state	"dome"-shaped colony morphology; high cloning efficiency; XaXa; germline chimera
		2i	(Ying et al, 2008) ground state	"dome"-shaped colony morphology; high cloning efficiency; XaXa; germline chimera
	Intermediate (formative)	FGF2/ Activin/ KSR (1%)	(Ohinata et al, 2009) (EpiLCs)	transient; high PGC induction efficiency
		FGF2/ Activin	(Han et al, 2010) (Oct4-GFP+EpiSCs)	chimera without germline contribution
		FGF4/ Serum	(Joo et al, 2014) (FGF4-EpiSCs)	XaXi; chimera without germline contribution
		FGF2/ Activin /CH	(Tsukiyama & Ohinata, 2014) (INTPSCs)	germline chimera
		FGF2/ Activin /IWP2	(Kurek et al, 2015) (IWP2-EpiSCs)	chimera without germline contribution
	primed	FGF2/ Activin	(Brons et al, 2007; Tesar et al, 2007) (EpiSCs)	"flattened" colony morphology; XaXi; little to no blastocyst chimera; broad engraftment to late epiblast
		FGF2/IWR1/ Serum free	(Wu et al, 2015) (rsEpiSCs)	XaXi; no blastocyst chimera; posterior biased engraftment to late epiblast

Human	totipotent-like	LCDM	(Yang et al, 2017) (EPS cells)	embryonic and extra-embryonic lineage contributions; interspecies chimeric competency in mouse conceptuses
	heightened	transient treatment with BAP (BMP4, A83-01 and PD173074)	(Yang et al, 2015)	sensitive to high FGF2 concentration; trophoblast differentiation in absence of BMP4 treatment
	naive	2i/LIF + DOX PD/CH/LIF/ FK	(Hanna et al, 2010)	transgenes-dependent: Klf4/Oct4 or Klf4/Klf2; “dome”-shaped colony morphology; XaXa; LIF-dependent
		NHSM	(Gafni et al, 2013)	high cloning efficiency; ;XaXa; DNA hypomethylation; de novo derivation from human blastocyst interspecies chimeric competency
		PD/BIO/ DOR/ LIF	(Chan et al, 2013)	LIF-dependent; coexpression of Gata6 and Nanog interspecies chimeric competency
		PD/CH/F GF2 or PD/CH/SU/L IF	(Ware et al, 2014)	XaXa; de novo derivation from human blastocyst (low efficiency); high cloning efficiency
		5iLA	(Theunissen et al, 2014)	de novo derivation from human blastocyst; XaXi; elevated and homogeneous expression of Nanog, Klf4, and Rex1; reduced level of H3K27me3
		PD/CH/LIF/ FGF2	(Wang et al, 2014)	HERVH+; XaXa; high cloning Efficiency
		FGF2/LIF/ PD/CH/FK/A A	(Duggal et al, 2015)	high cloning efficiency; XaXa; DNA hypomethylation
	primed	FGF2/KSR; Serum; mTeSR	(Ludwig et al, 2006; Reubinoff et al, 2000; Thomson et al, 1998)	“flattened” colony; XaXi; low cloning efficiency; late epiblast interspecies chimeric competency
		F2/IWR1/ Serum free	(Wu et al, 2015) (rsESC)	high cloning efficiency; XaXi; late epiblast interspecific chimeric competency

Table-1: Examples of the Different ‘States’ of Mouse and Human PSCs and Their Properties

CH, CHIR99021; KSR, Knockout Serum Replacement; DOX, Doxycyclin; PD; PD0325901; FK, Forskolin; DOR, Dorsomorphin; SU, SU5402; AA, Ascorbic Acid; 2i: CHIR99021 and PD0325901.

LCDM: LIF, CHIR99021, (S)-(+)-dimethindene maleate (DiM) and minocycline hydrochloride (MiH)

NHSM: LIF, TGFb1, FGF2, ERK1/2i (PD0325901), GSK3i (CHIR99021), JNKi (SP600125) and p38i (SB203580). 5iLA: LIF, PD0325901, IM-12, SB590885, WH-4-023, Y-27632, Activin-A.

T2ILGO: LIF, PD0325901, CHIR99021, Go6983. Adapted from(Wu & Izpisua Belmonte, 2015).

Molecular regulation of pluripotency

PSCs are characterized by molecular mechanisms that sustain self-renewal and suppress differentiation. The molecular regulation of pluripotency is governed by signaling pathways, transcription factors and epigenetic factors. The regulation of pluripotency in response to signaling cues is the main driver for cellular fates such as self-renewal and commitment. Additionally, deciphering the signaling pathways regulating pluripotency has permitted the utilization of recombinant proteins, small molecules and inhibitors that promote self-renewal and somatic cell reprogramming while inhibiting commitment. My thesis work led to identify of a new signaling molecule that regulates both self-renewal and commitment. For this purpose, in the next section, I describe the molecular regulators of pluripotency with a focus on signaling pathways.

Signaling pathways

LIF/STAT3 pathway

Signaling pathways maintain pluripotency by promoting proliferation and by inhibiting differentiation. ESCs, like EC cells, are first derived on MEFs (feeders). Afterwards, leukemia inhibitory factor (LIF) was identified as the principal cytokine secreted from MEFs to maintain self-renewal and inhibit differentiation of ESCs (Smith et al, 1988; Williams et al, 1988). Mouse ESCs can be cultured by addition of LIF in feeder-free condition.

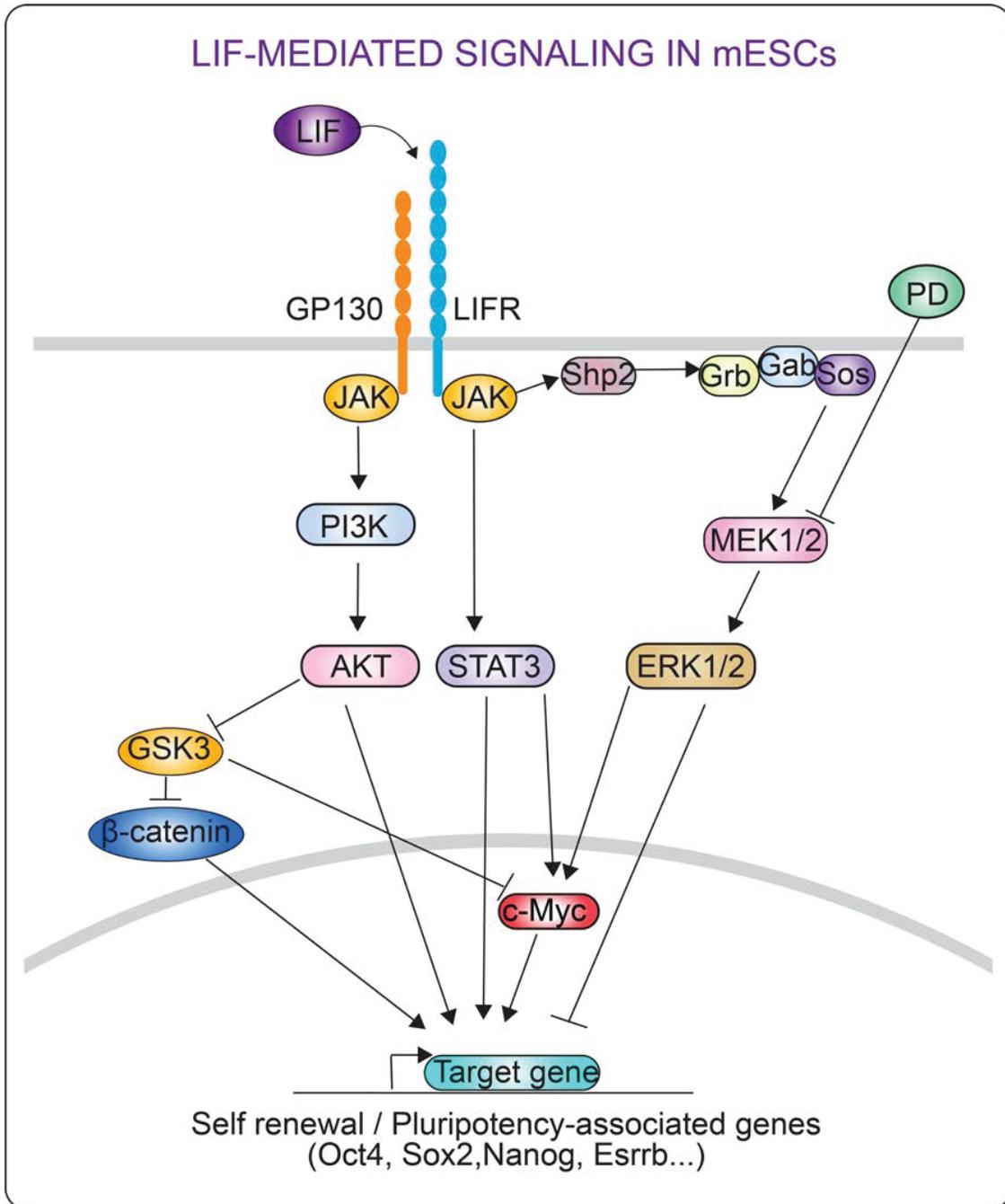


Figure-4: Schematic depiction of LIF-mediated signaling pathways in mESCs.

Association of LIF with its heteromeric receptor, which consists of LIFR and GP130, results in activation of the STAT3 pathway, the Ras/ERK pathway, and the PI3K/AKT pathway. Upon LIF binding to the receptor, JAKs are activated by auto-phosphorylation inducing downstream cascades. STAT3 is phosphorylated and activated by JAKs, and subsequently induces the expressions of various pluripotency-associated genes and c-Myc. Activated PI3K/AKT by JAKs also positively regulates the genes, and stabilizes c-Myc via inhibiting GSK3 activity. In contrast to these 2 pathways, activated ERK1/2 pathway inhibits the expressions of the pluripotency-associated genes but positively regulates c-Myc. ERK1/2 activity can be inhibited by PD (PD0325901).

LIF is a member of the Interleukin 6 (IL6) family of cytokines. The heteromeric receptor complex for LIF is composed of LIF Receptor (LIFR) and GP130 (Zhang et al, 1997). Upon ligand binding, Janus Kinase (JAK) phosphorylates tyrosine residues of LIFR and GP130. Following receptor phosphorylation, the Signal Transducers and Activators of Transcription (STAT) 1 and STAT3 are recruited to the receptor complex and are phosphorylated by JAK. Upon dimerization STATs translocate into nucleus to activate transcription (Stahl et al, 1995). STAT3 is identified as a key mediator of mouse embryonic stem cell self-renewal (Niwa et al, 1998) and STAT3 activation is sufficient for self-renewal in the presence of Fetal Bovine Serum (FBS) (Matsuda et al, 1999). However STAT3 is not strictly sufficient for self-renewal without additional presence of FBS (Ying et al, 2003) **(Figure-4)**.

In addition JAK/STAT3 signaling promotes mouse somatic cell reprogramming (Tang et al, 2012). However the role of JAK/STAT3 signaling in mouse reprogramming to a naïve state is controversial. While an initial publication suggests that, JAK/STAT3 signaling is a limiting factor for reprogramming to naïve ground state (Yang et al, 2010), the researchers reported only two years later that JAK/STAT3 signaling is sufficient and dominates differentiation cues in reprogramming to naïve pluripotency (van Oosten et al, 2012).

Unlike mouse ESCs, human ESCs maintenance is not STAT3 dependent (Humphrey et al, 2004). However, recently Chen et al. discovered that reinforcement of STAT3 activity reprograms human ESC to naïve pluripotency (Chen et al, 2015). Moreover LIF is used in several different cocktails inducing a naïve state of hPSCs (Gafni et al, 2015; Guo et al, 2016; Hanna et al, 2010; Ware et al, 2014).

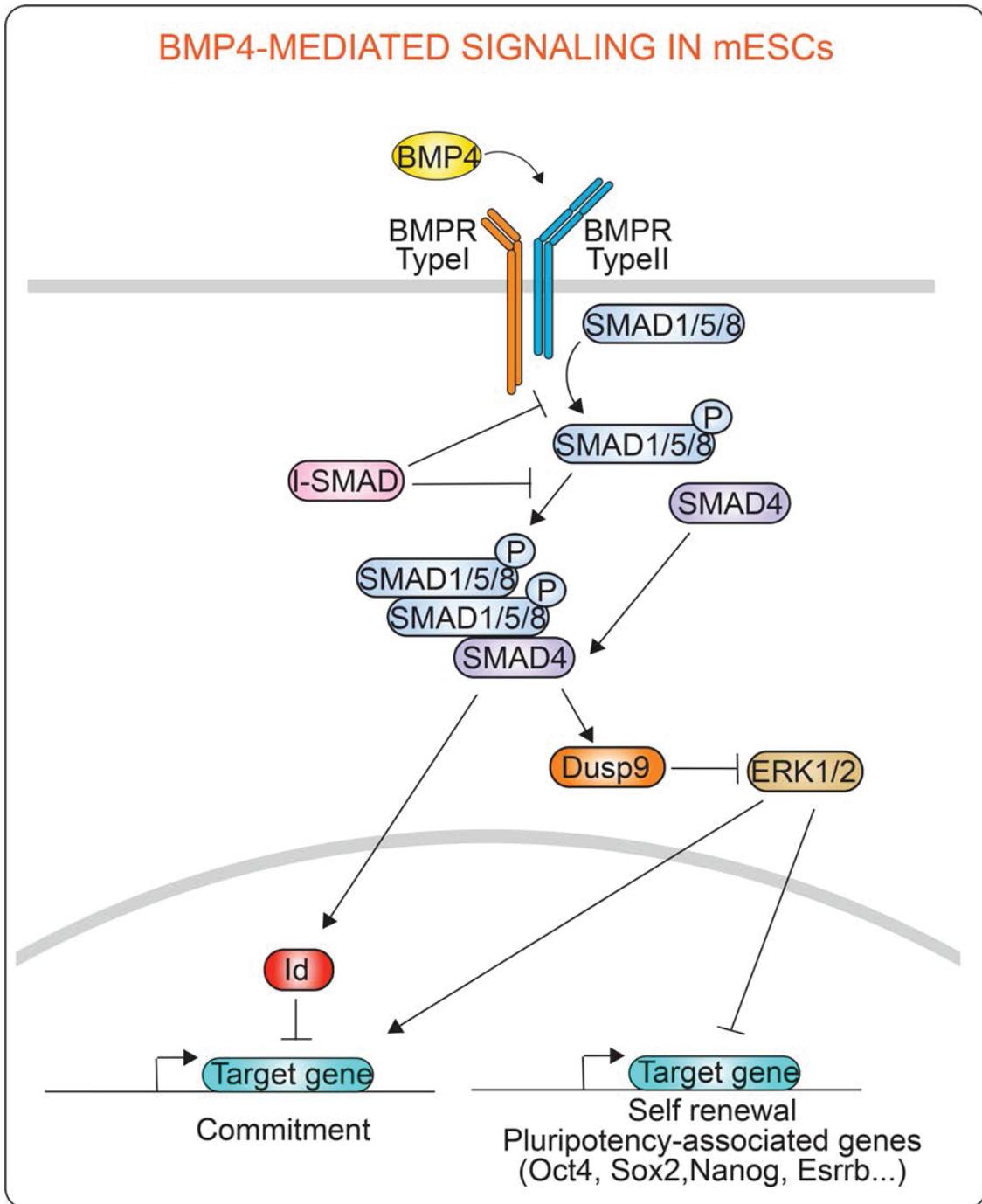


Figure-5: Schematic depiction of BMP4-mediated intra-cellular signaling pathways in mESCs. Association of BMP4 with its receptors, a heteromeric complex of type I and type II receptor serine/threonine kinases, induces phosphorylation of Smad1/5/8. Phosphorylated SMAD1/5/8 form complexes with Smad4 and accumulate in the nucleus, where together they regulate gene transcription. The SMAD complexes induce Id expression to inhibit differentiation, and they suppress ERK1/2 signaling via Dusp9. I-Smads suppress the pathway via more than one mechanism, as shown.

Bone Morphogenetic Protein (BMP) is a member of the Transforming Growth Factor β (TGF- β) superfamily and binds to heterodimeric receptor complexes composed of type I and type II receptors. Ligand binding, and subsequent formation of receptor complexes cause phosphorylation of SMADs. SMAD complexes then translocate to nucleus and act as transcription factors (Heldin et al, 1997). BMP4 treatment suppresses neuronal differentiation by inducing expression of inhibitor of differentiation (Id) factors (*reviewed in* (Ruzinova & Benezra, 2003)). BMP4, in combination with LIF, is sufficient to maintain mouse ESC pluripotency in feeder- and serum-free culture conditions (Ying et al, 2003). Moreover, through DUSP9-mediated inhibition of ERK activity, BMP signaling reinforces the self-renewal status of mESCs together with LIF (Li et al, 2012) **(Figure-5)**.

In addition BMP-SMAD signaling facilitates mouse iPSC generation (Samavarchi-Tehrani et al, 2010). In human ESC, BMP signaling induces differentiation and inhibition of BMP supports self-renewal (Schuldiner et al, 2000; Xu et al, 2005). However, Hayashi et al. showed that BMP4 activation promotes human iPSC generation by inhibiting senescence (Hayashi et al, 2016).

WNT/ β -catenin pathway

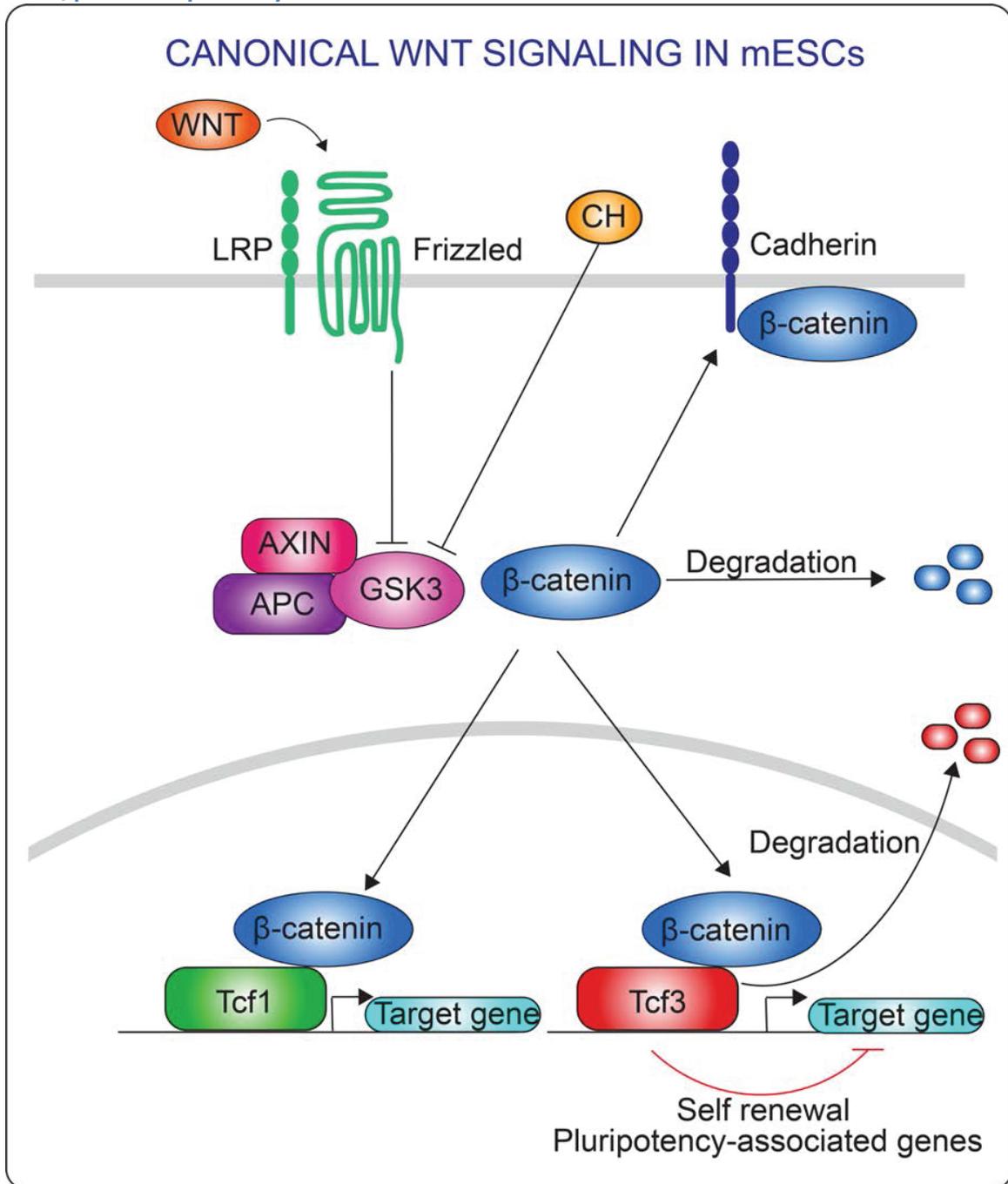


Figure-6: Schematic depiction of canonical WNT signaling pathway in mESCs.

WNT binds to its receptor, Frizzled, and coreceptor, Lrp (5/6) and causes GSK3 inhibition. GSK3 can also be inhibited by application of an inhibitor, such as CH (CHIR99021). GSK3 induces degradation of β -catenin in the absence of WNT ligand. GSK3 inhibition results in β -catenin recruitment to cell membranes, where it associates with E-cadherin, or in β -catenin localization to the nucleus. Nuclear β -catenin associates with Lef1 and Tcf1 to activate their transcriptional activator function. β -catenin also binds to Tcf3 and inhibits its repressor function via degradation.

The soluble factor WNT was reported to maintain mouse and human pluripotency (Sato et al, 2004). WNT receptor is a heteromeric complex composed of Frizzled and Lrp5/6. In the absence of WNT, a destruction complex consisting of Axin, APC, and Glycogen Synthase Kinase 3 (GSK3) resides in the cytoplasm, where it binds to and phosphorylates β -catenin, targeting it for degradation. GSK3- β inhibition by WNT stimulation or by GSK3- β inhibitor such as CH (CHIR99021) involves interaction between Axin with Lrp5/6 and inhibition of the destruction complex. GSK3- β inhibition results with stabilization and nuclear translocation of β -catenin where it interacts with transcription factors, the members of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family, to activate transcription (Clevers et al, 2014).

Nuclear β -catenin triggers the activator function of Tcf1 and Lef1 (Merrill et al, 2001; Wu et al, 2012). β -catenin also binds to Tcf3 and subsequently causes its degradation by proteasome and inhibits its repressor function. This is important, as Tcf3 interacts with and represses core pluripotency-associated transcription factors, nuclear β -catenin translocation stabilizes pluripotency (Shy et al, 2013). Stabilized β -catenin can also localize to the cellular membrane and membrane-associated- β -catenin promotes ground state pluripotency (Faunes et al, 2013). Smith and colleagues previously demonstrated that in a serum-and feeder-free defined medium, GSK3 inhibitor combined with MEK inhibitor or with the addition of LIF is sufficient to support long-term pluripotency maintenance (Ying et al, 2008). The combination of exogenous LIF, ERK inhibitor with GSK inhibitor or WNT3a stimulation together stimulated the most vigorous self-renewal of mESCs (Silva et al, 2008; ten Berge et al, 2011; Ying et al, 2008) **(Figure-6)**.

WNT signaling also regulates differentiation abilities of mESCs. Homozygous APC mutant mESCs or double knockout GSK3 (for GSK3 α and GSK3 β) mESCs were not able to truly differentiate in teratoma formation and embryoid body formation assays. They retained high levels of pluripotency transcription factors (Oct4, Nanog,

Rex1) levels during differentiation (Doble et al, 2007; Kelly et al, 2011; Kielman et al, 2002).

Human ESCs self-renewal is not stimulated by WNT signaling (Dravid et al, 2005). Similarly, the use of the GSK inhibitor alone induced differentiation of mEpiSCs. However, GSK inhibition together with LIF and MEK inhibitor induced conversion of mEpiSCs into mESCs (Greber et al, 2010). Moreover, GSK3 inhibitor, together with other supplements and inhibitors, promotes naïve pluripotency in human (Gafni et al, 2015; Guo et al, 2016; Hanna et al, 2010; Ware et al, 2014).

WNT signaling also positively regulates mouse and human somatic cell reprogramming (Marson et al, 2008; Ross et al, 2014). Together this data suggest that WNT activation and/or GSK3 inhibition alone stimulates differentiation of primed cells but promotes self-renewal of naïve pluripotent stem cells.

PI3K pathway

Phosphatidyl inositol 3 Kinase (PI3K) is induced by many different receptor tyrosine kinases for growth factors (such as FGF, Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF)) or upon LIF stimulation (Paling et al, 2004). PI3K induction results in the radial generation of second messenger PIP3 that results in AKT activation. Activated AKT is a highly pleiotropic effector that modulates cellular responses via Mouse Double Minute 2 homolog (MDM2), IκB Kinase (IKK) and mechanistic target of rapamycin (mTOR), among others (*reviewed in* Okita & Yamanaka, 2006). The PI3K pathway promotes mESCs proliferation (Sun et al, 1999), maintains mouse and human pluripotent cell self-renewal and survival (Hossini et al, 2016; Paling et al, 2004).

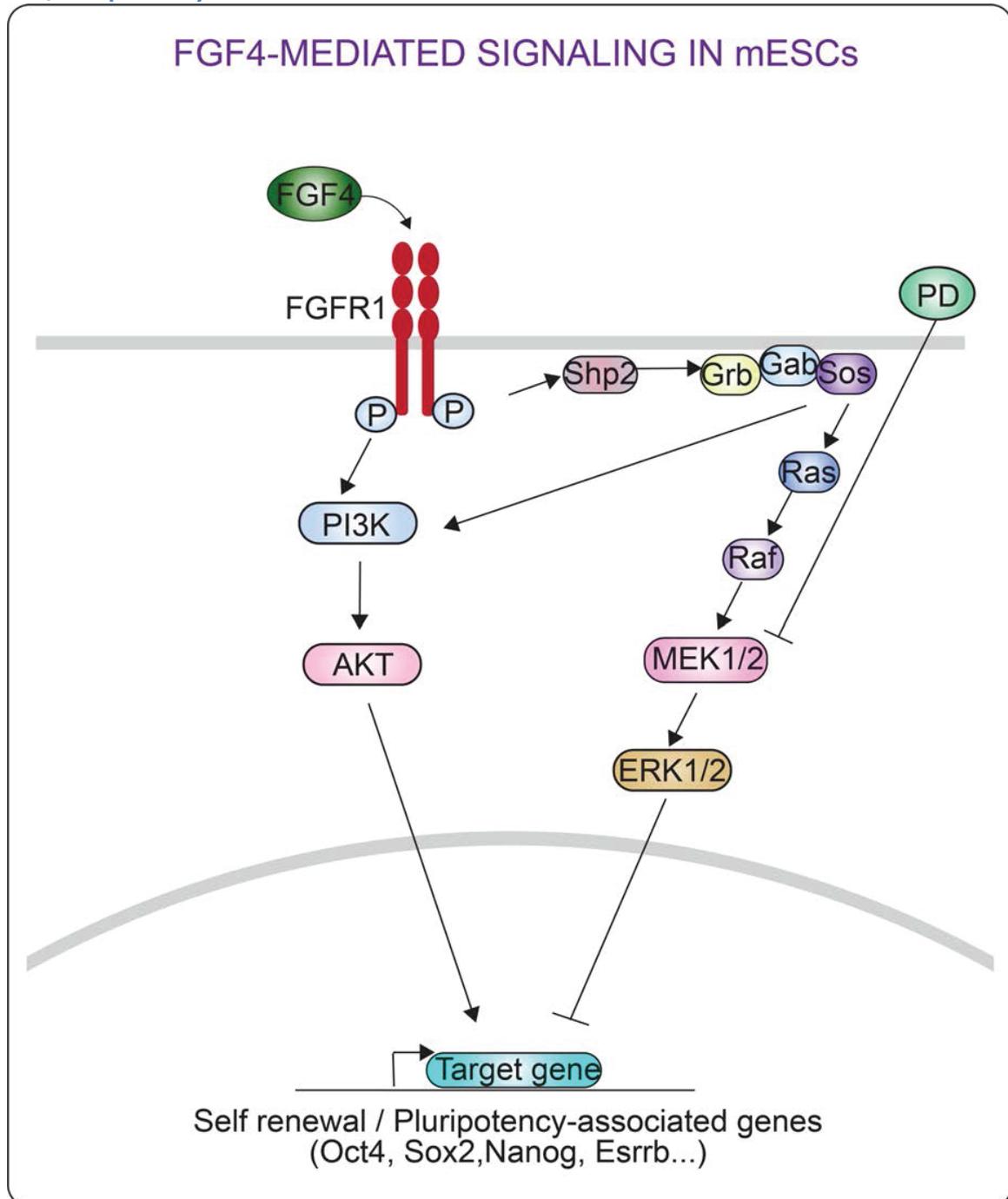


Figure-7: Schematic depiction of FGF4 signaling pathway in mESCs.

FGF4 binding to FGFR1 receptor (the sole FGF receptor present in mESCs) induces autophosphorylation of receptors and phosphorylation of receptor-associated proteins. The activated Ras, Raf, MEK1/2 and ERK1/2 induce mESCs differentiation. The receptor-ligand complex activation also induces PI3K/AKT pathway.

Ras proteins are GTP-binding proteins that are linked to cell proliferation. Many different growth factors (such as FGF) or LIF can activate Ras. Activated Ras binds to effector proteins including Raf serine/threonine kinases, which activates ERK/MAPK. FGF4 is the main cue secreted from mESCs in an autocrine manner and induces mESCs to exit self-renewal and initiate differentiation (Kunath et al, 2007). **(Figure-7)** Furthermore, chemical inhibition or genetic knockout of the FGF/ERK signaling cascade components caused mESC differentiation defects (Burdon et al, 1999; Kunath et al, 2007). Mitogen Activated Protein Kinase Kinase (MEK) inhibitor is used to grow both human naïve and mouse naïve PSCs (Gafni et al, 2013; Wray et al, 2010; Ying et al, 2008).

These signaling pathways are the main regulators of pluripotency and commitment. The crosstalk between them justifies the use of different signaling modulators together such as PD, CH and LIF in stem cell media for pluripotency maintenance **(Figure-8)**.

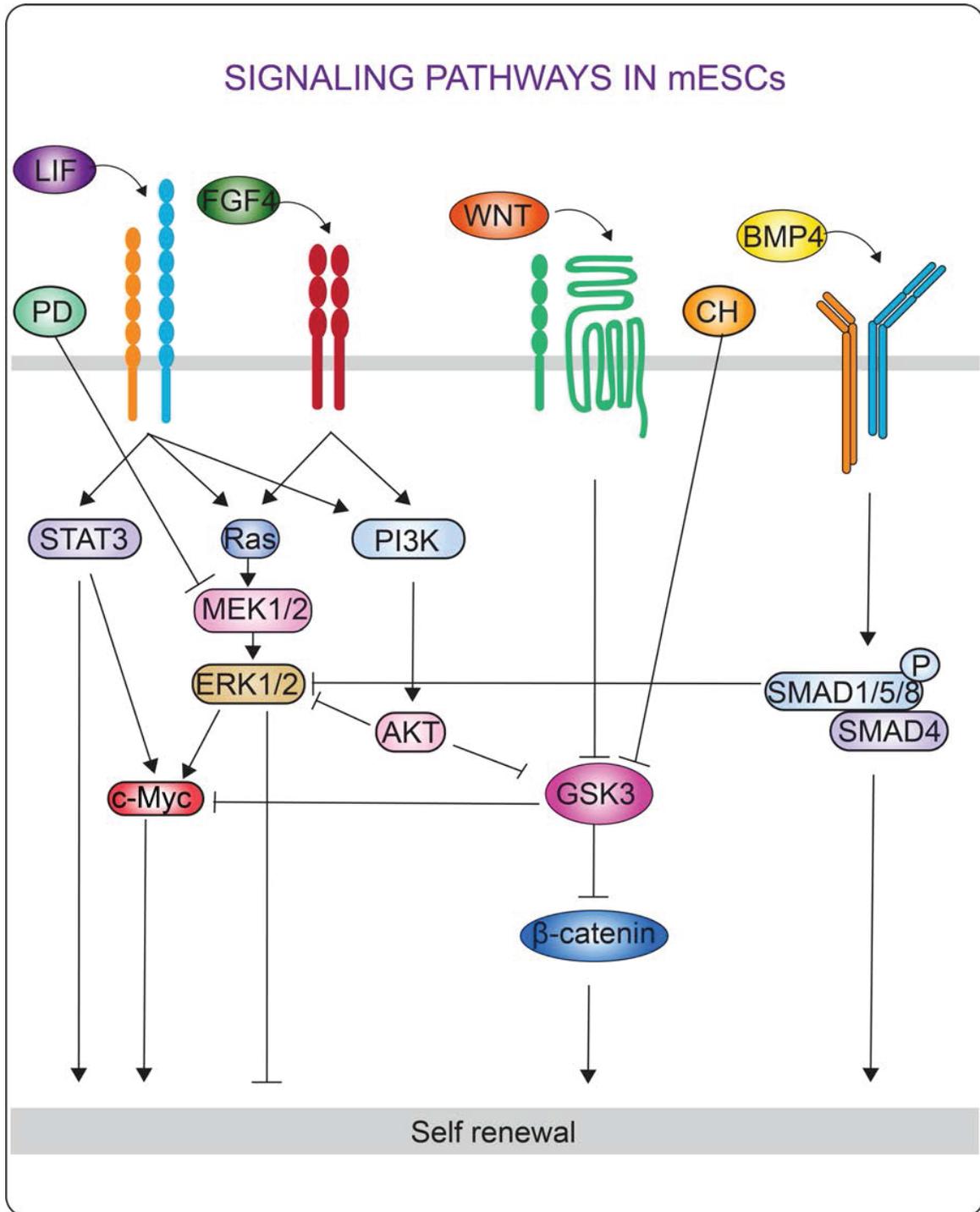


Figure-8: Schematic representation of crosstalk between signaling pathways in mESCs. LIF, WNT and BMP4 signaling pathways in mESCs induce self-renewal and maintain pluripotency. FGF4 induces commitment. PD and CH are used with or without LIF for ground state mESCs.

Transcription factors

A set of transcription factors governs and defines pluripotency. In addition to the transcription factors, the epigenetic regulators have been well recognized as critical mediators of pluripotency. In this chapter, main transcription factors and epigenetic regulators of pluripotency maintenance and somatic cell reprogramming are described.

Core transcription factors: Oct4/ Sox2/ Nanog

Transcription factors (TFs) maintain pluripotency in response to (or downstream of) extracellular cues and related signaling pathways. In ESCs Oct4, Sox2 and Nanog are core transcription factors maintaining pluripotency. The core transcription factors function together to positively regulate their own promoters forming an auto-regulatory loop and co-occupy and activate expression of genes to maintain the ESC state, while contributing to repression of genes guiding exit from the pluripotent state (*reviewed in* (Young, 2011)).

The POU family transcription factor Oct4, encoded by *Pou5f1* gene, is a central regulator of pluripotency (Nichols et al, 1998; Scholer et al, 1990). Oct4 is expressed within totipotent mouse blastomeres, pluripotent epiblast and primordial germ cells (Yeo & Ng, 2013). Inducible suppression of an Oct4 transgene induces differentiation towards trophectoderm (TE) lineage, whereas overexpression of Oct4 induces differentiation towards primitive endoderm-like cells and promotes mesendodermal differentiation of ESCs (Niwa et al, 2000; Thomson et al, 2011). In pre-implantation embryos, Oct4 is co-expressed and forms a co-repression complex with the trophectoderm transcription factor Caudal Type Homeobox 2 (Cdx2). This complex mediates reciprocal repression; increased expression of one factor reduces

the activity of the other, ensuring that the dominant activity in a given cell will promote differentiation to TE cells (Cdx2 dominant) vs pluripotent cells (Oct4 dominant) (Niwa et al, 2005). Unexpectedly, mESCs with reduced Oct4 expression resulting from heterozygosity (Oct4 +/-) exhibit a stabilized pluripotent state by increasing genome wide binding of Oct4, particularly at pluripotency-associated enhancers. Oct4 +/- cells also display homogeneous expression of pluripotency TFs, enhanced self-renewal and delayed differentiation kinetics, increased WNT expression, enhanced LIF sensitivity and reduced responsiveness to FGF (Karwacki-Neisius et al, 2013). Altogether these data indicate that Oct4 expression levels are tightly regulated and its interaction with other TFs and/or enhancer regions controls differentiation, pluripotency maintenance and dictates distinct pluripotency states.

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encodes TFs with a single HMG DNA-binding domain. Sox2 is expressed within ICM, extraembryonic endoderm of pre-implantation blastocysts (Avilion et al, 2003), in trophoblast (Keramari et al, 2010) and in neural precursors (Ferri et al, 2004). Sox2 protein physically interacts with Oct4 (Pardo et al, 2010) and Oct4-Sox2 complex binds to DNA and activate Oct-Sox enhancers (Chen et al, 2008). Cooperation between Oct4 and Sox2 was first observed during the activation of the FGF4 enhancer and then of several ES-specific enhancers (Niwa, 2001; Tomioka et al, 2002; Yuan et al, 1995). Sox2 deletion, similarly to Oct4, causes ESCs differentiation to throphoectoderm-like cells and the differentiation default of Sox2 deleted cells can be rescued by forced expression of Oct4. The rescued-cells activated Oct-Sox enhancers but did not contribute chimera formation (Masui et al, 2007). Moreover, overexpression of Sox2 induces neural ectodermal differentiation (Kopp et al, 2008; Thomson et al, 2011). These results together suggest other roles for Sox2 in pluripotency regulation in addition to activation of Oct-Sox enhancers and indicate that Sox2 levels, such as Oct4 levels, must be strictly regulated for pluripotency maintenance.

Nanog, the homeodomain-containing transcription factor, was identified by two independent screens (Chambers et al, 2003; Mitsui et al, 2003). Nanog expression is more restricted to naïve epiblast *in-vivo* compared to Oct4 and Sox2 (Silva et al, 2009) and is re-expressed during gastrulation until being lost at the onset of somitogenesis. Nanog is also present in epiblast-derived mESCs and in primordial germ cells (PGCs) of mammals and birds (*reviewed in* (Festuccia et al, 2013))(Laval et al, 2007). Gene expression and lineage-tracing experiments have shown that the early ICM already contains distinct subpopulations of cells that selectively express the TFs Nanog or GATA binding factor 6 (GATA6) in a position-independent, random and mosaic ‘salt and pepper’ pattern, and are committed to become either epiblast or primitive endoderm (PE), respectively (Chazaud et al, 2006). Cells retaining Nanog proceed to complete transcriptional and epigenetic resetting including reactivation of the inactive paternally inherited X chromosome in females (Silva et al, 2009). Forced expression of Nanog in ES cells confers self-renewal ability in the absence of LIF (Chambers et al, 2003). Conditional gene deletion in ES cells revealed that Nanog is not essential for propagation of pluripotency *ex vivo* but destabilize clonogenicity of pluripotent cells (Chambers et al, 2007), however Nanog null ESCs and embryos fail to generate epiblast (Mitsui et al, 2003) (Silva et al, 2009).

Even if Oct4, Sox2 and Nanog are accepted as core regulators of pluripotency functioning together, they do not regulate pluripotency in the same manner. While Oct4 and Sox2 overexpression leads to differentiation, Nanog overexpression renders ESCs LIF independent. Moreover, genome-wide studies show that Nanog co-occupies only some regions that are bound by Oct4-Sox2 and has differential binding sites compared to Oct4-Sox2 binding sites (Loh et al, 2006). These findings suggest that Nanog has partially shared and also complementary regulatory activity compared to Oct4-Sox2. Moreover, within 3 core transcription factors, only Nanog belongs to ‘ground state transcription factors’. It is also suggested that all pluripotency transcription factors are lineage specifiers that direct commitment to specific lineages since overexpression of many pluripotency regulators induce differentiation towards a lineage. This model suggests that pluripotency factors

cooperatively block differentiation into all lineages (Loh & Lim, 2011).

Ground State & Naïve epiblast Transcription Factors: Nanog/ Esrrb/ Klf2/ Klf4/ Tbx3

Transcription factor heterogeneity has been described for a set of gene in conventional cell culture conditions without feeders or 2i. Such heterogeneity is shown for transcription factors such as Rex1 (Zfp42), Nanog, Kruppel Like Factor 2 (Klf2), Klf4, T-Box3 (Tbx3) and Estrogen Related Receptor Beta (Esrrb). (Smith, 2013). These factors are called 'ground state factors' because they are strongly expressed in ground state conditions *in-vitro* and in naïve epiblast *in-vivo*. They are downregulated upon post-implantation. Among ground state factors, sustained expression of Esrrb, such as Nanog, can render ESCs LIF independent even though Esrrb is not a target of LIF signaling(Festuccia et al, 2012). Furthermore Nanog, Klf2, Esrrb are targets of the repressor Tcf3 and their expression is increased upon GSK3 inhibition. Moreover sustained expression of Esrrb also renders cells GSK3i independent(Martello et al, 2012). These findings suggest that these transcription factors regulate pluripotency through various signaling pathways and parallel mechanisms. Dunn et al., suggest a simple model composed of 16 interactions, 12 components and 3 inputs (LIF, CH and PD) and proposed that the extrinsic cues regulate distinct transcription factors that are interconnected to stabilize naïve pluripotency (Dunn et al, 2014). However this model is established upon only known extrinsic cues regulating pluripotency till today. Future discoveries identifying new extrinsic cues regulating self-renewal and commitment can lead a better understanding of pluripotency.

A transcription factor with pleiotropic functions: Myc

The Myc family of transcription factors comprises c-Myc, N-Myc, and L-Myc. Myc is not a classical pluripotency transcription factor. Myc has pleiotropic functions in various biological contexts. Myc regulates embryonic stem cell self-renewal and

pluripotency, iPSCs reprogramming, cellular proliferation, inhibition of differentiation, cellular adhesion and various aspects of tumorigenesis (Adhikary & Eilers, 2005; Cartwright et al, 2005; Takahashi & Yamanaka, 2006). Within Myc family, c-Myc is the most studied factor in PSCs. C-Myc expression is a common target of LIF/STAT3, WNT/ β -catenin and Ras/ERK pathways. LIF/STAT3 induce c-Myc expression to promote self-renewal and pluripotency maintenance (Cartwright et al, 2005). Moreover, Ras/ERK pathway enhances Myc protein stability (Sears et al, 1999). GSK3 β negatively regulates c-Myc activity by phosphorylation following degradation by the proteasome system (Sears et al, 2000). Thus c-Myc is a common target both LIF and WNT signaling pathways. In ESCs, one third of the promoters of active genes are bound both by c-Myc and by core transcription factors, Oct4/Sox2 and Nanog (Rahl et al, 2010). However, c-Myc and core transcription factors functions in a different manner for gene regulation. Core transcription factors select ESC genes for expression through the recruitment of RNA Pol II, while c-Myc functions to control gene expression through the release of transcriptional pause (Young, 2011). Moreover, it is suggested that c-Myc acts only to amplify the existing transcriptional output of active genes rather than activating new genes (Nie et al, 2012). This result indicates that c-Myc is unable to initiate *de novo* gene activation. This ability to amplify existing active gene transcription may suggest how the addition of c-Myc is able to increase the efficacy of iPSCs formation (Nakagawa et al, 2008). In iPSCs reprogramming, reactivation of c-Myc retrovirus results in increased tumorigenicity in the chimeras and progeny mice, hindering clinical applications. However, the protocols enabling iPSCs generation without retrovirus induced c-Myc has been described (Nakagawa et al, 2008) and are important for clinical applications.

Epigenetic Regulators

Beside the signaling pathways and transcription factors; cofactors, chromatin regulators and histone, DNA and chromatin modifiers contribute to pluripotency regulation and maintenance and cellular reprogramming.

Cofactors

Cofactors are proteins that mediate repressor or activator response but they don't have DNA-binding capacity. The cofactors, mediator and cohesion, physically and functionally connect to the enhancers and to the promoters of active genes in ESCs to regulate pluripotency and ESCs self-renewal (Fazio & Panning, 2010; Kagey et al, 2010). The Tip60-p400 complexes, Cnot3, Trim28, Paf1, Dax1, Tex10, Cbfa2t2 are identified as important regulators of pluripotency maintenance (reviewed in(Li & Belmonte, 2017)). Moreover, Ncoa3 is another important example of cofactors by bridging ESCs TF Esrrb to RNA polymerase II complexes (Percharde et al, 2012) to activate transcription for the maintenance of ESCs self renewal and somatic cell reprogramming.

RNA-based regulators of pluripotency

MicroRNAs

MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that regulate gene expression by post-transcriptional RNA silencing. They are accepted as important regulators of pluripotency and reprogramming (Choi et al, 2011; Choi et al, 2017b; Judson et al, 2009). Recent studies identifies that ESC-specific miRNAs promotes self-renewal and inhibit differentiation. DCGR8 deficient ESCs can't exit pluripotency and differentiate poorly (Wang et al, 2007). These findings suggest that miRNA biogenesis is dispensable for pluripotency but required for lineage commitment.

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) have emerged as another important regulators of pluripotency. Several lncRNAs have been identified as targets of Oct4 and Nanog and modulators of pluripotency-associated gene expression and differentiation (Sheik Mohamed et al, 2010). A large intergenic non-coding RNA (lincRNA) regulator of reprogramming (lincRNA-ROR) is identified as a modulator of human iPSCs reprogramming (Sheik Mohamed et al, 2010).

Alternative splicing

Alternative splicing broadens proteome diversity by allowing multiple protein isoforms. Specific isoforms of pluripotency factors such as Forkhead Box P1 (Foxp1), Nanog and Mbd2 have been shown to maintain pluripotency and favors iPSCs reprogramming (Das et al, 2011; Gabut et al, 2011; Lu et al, 2014b).

Histone modifications

Pluripotent stem cells have distinct profiles of histone modifiers and modified histones. In PSCs, Trithorax group (TrxG) complexes catalyze trimethylation of H3K4 at the transcriptionally promoters of active genes and promote ESCs self-renewal and somatic cell reprogramming (Ang et al, 2011). The methylation of H3K4 is mediated by TrxG members such as Set/MLL methyltransferases (reviewed in (Watanabe et al, 2013)). Wdr5, a key component of TrxG, interacts with Oct4 and regulates Oct4 expression levels to promote ESCs self-renewal and somatic cell reprogramming (Ang et al, 2011). In PSCs, Polycomb group (PcG) complexes occupy developmental genes that must be repressed to maintain pluripotency and that are poised for rapid activation during differentiation (Lee et al, 2006). H3K27 methylation is mediated by polycomb repressive complex 2 (PRC2). PRC2 is

composed of Ezh2, Eed and Suz12. mESCs lacking a single member of PRC2 complex show disrupted self-renewal and complete loss of H3K27me3 (Boyer et al, 2006; Chamberlain et al, 2008). In hESCs, Suz12-bound and H3K27me3-enriched genes are co-occupied with Oct4, Sox2 and Nanog. The canonical PRC1 complexes composed of Ring1a, Ring1b, Bmi1 and Mel18 are also involved in the repression of transcription of developmental genes (Jorgensen et al, 2006).

Transcriptionally inactive heterochromatin is usually accompanied by H3K9 di- and tri- methylation in pluripotency maintenance. Particularly, the H3K9 methyltransferase SETDB1 contributes repression of developmental regulators (Bilodeau et al, 2009). The acetylation of histones is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) and is also a feature of pluripotent stem cells. The level of acetylation is generally correlated with transcriptional activation. The HAT complex Tip60/p400 is a regulator of pluripotency and is localized both on active and silent gene promoters (Fazzio et al, 2008).

The promoters of many different genes of ESCs harbor both active (H3K4me3) and repressive (H3K27me3) histone marks, a 'bivalent state' thought to facilitate activation of development genes upon exit from pluripotency (Azuara et al, 2006; Bernstein et al, 2006). This 'bivalent state' is observed mainly at promoters of lineage-specific genes suggesting that the genes in bivalent domains are poised for activation during differentiation but they are kept silent by the mark H3K27me3 in ESCs (Azuara et al, 2006; Bernstein et al, 2006). Interestingly, in ground state culture conditions (2i) compared to conventional culture conditions (serum+LIF), H3K27me3 and PRC2 complex members are much less present (Marks et al, 2012). Also ~2000 genes with bivalent domains in serum+LIF, are not bivalent in 2i conditions (Marks et al, 2012). These results indicate that the epigenome of ground state mESCs is distinct and presence of bivalency is positively correlated with heterogeneous pluripotent stem cells. This finding also suggests that bivalency is required to repress lineage-specific genes that are slightly expressed in

conventional culture conditions.

DNA methylation and demethylation

DNA methylation is an important regulator of pluripotency and a barrier for reprogramming. During embryogenesis DNA methylation is highly dynamic and established by the DNA methyltransferase Dnmts (Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L). Upon fertilization DNA methylation is gradually decreases and reaches a minimum level in blastocyst stage (Smith et al, 2012). After implantation, DNA methylation increases and it is thought to play an important role in lineage restriction and loss of potency (Smith et al, 2012). *In-vitro*, 2i ESCs have lower levels of Dnmt3a, Dnmt3b and Dnmt3L than serum+LIF ESCs and all 3 Dnmts increase when they are transferred from 2i to serum+LIF condition (Leitch et al, 2013). This finding suggests, like loss of 'bivalency' of histones, DNA methylation is also decreased in ground state conditions. Moreover, DNA methylation is required to exit from naïve pluripotency and for robust embryonic development.

On the other hand, the DNA demethylase, Ten-eleven-translocation (Tet) is highly expressed in mESCs, and down regulated upon cell differentiation (Ito et al, 2010). However Tet levels remain similar *in-vitro* in 2i and serum+LIF conditions (Habibi et al, 2013). This result suggests loss of Dnmts is more critical than Tet activity for ESCs homogeneity.

Other chromatin remodelers

Methyl-CpG-binding domain protein 3 (Mbd3) / Nucleosome Remodeling Deacetylase (NURD) complex is identified as a controller of Tet1 target gene expression in ESCs (Habibi et al, 2013). The NURD complex includes Chd3, Chd4, Mta1, Mta2, Mbd3, and is responsible for H3K27 deacetylation and trimethylation.

In addition NURD complex is essential for pluripotency maintenance and early embryogenesis (Kaji et al, 2006; McDonel et al, 2009; Reynolds et al, 2012). Moreover, Mbd3/NURD complex is identified as a barrier for deterministic reprogramming (Rais et al, 2013)

SWItch/Sucrose Non-Fermentable (SWI/SNF) complexes trigger ejections of nucleosomes and composed of BAF and PBAF complexes. They contribute self-renewal and lineage commitment of ESCs (Gao et al, 2008).

CHAPTER II: NETRIN-1

This chapter of the thesis introduces the guidance cue Netrin-1 and its receptors. During my PhD, I identified Netrin-1 and its receptors as new regulators of reprogramming and pluripotency. In this chapter, I describe the discovery, function and signaling of Netrin-1 and its receptors in different physiological and pathological contexts.

Netrin-1 and its receptors

Discovery and evolution of Netrins – the “one who guides”

UNC-6/Netrin is described for the first time as a guidance cue in *C. elegans* mutants exhibiting an uncoordinated (Unc) phenotype with axon guidance defects (Hedgecock et al, 1990; Ishii et al, 1992). Secreted netrins have been found in several invertebrates and vertebrates. These animals exhibit origins of bilateral symmetry, which is the vertical division of the body into left and right halves, and direct axonal migration in developing nervous system (de la Torre et al, 1997; Harris et al, 1996; Matus et al, 2006; Mitchell et al, 1996). These findings indicate that the roles of netrins have been conserved during evolution. To date, in mammals three secreted netrins, Netrin -1, -3, -4, and two membrane- tethered netrins, NetrinG1 and NetrinG2, have been identified (Koch et al, 2000; Nakashiba et al, 2000; Nakashiba et al, 2002; Serafini et al, 1996; Van Raay et al, 1997) **(Figure-9)**.

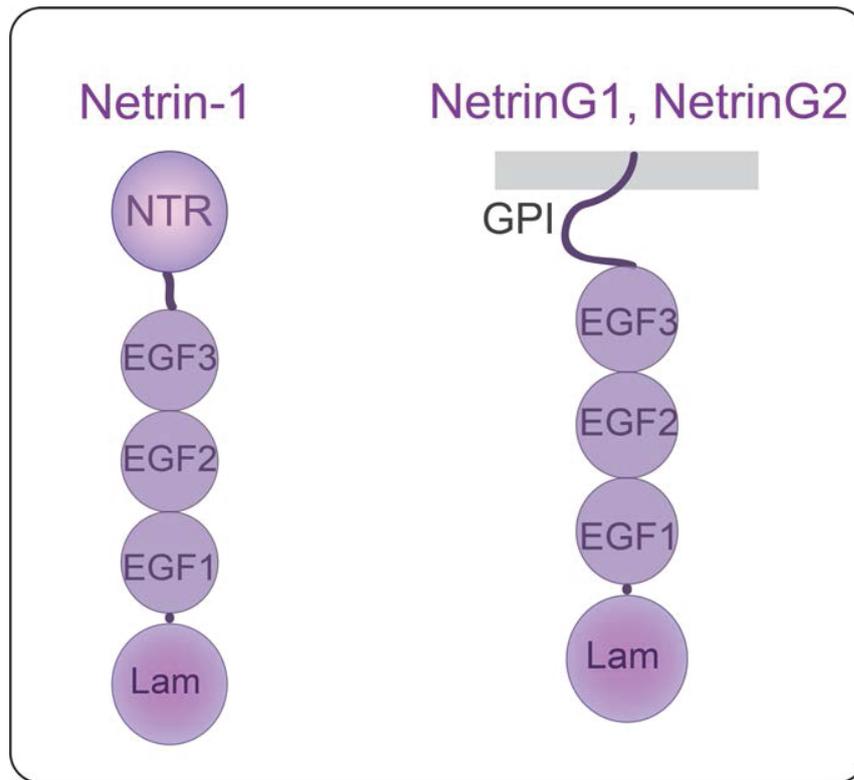


Figure-9: Structure of Netrin-1/3/4, NetrinG1 and NetrinG2

All Netrins are composed of an N terminal Laminin (Lam) (LN) domain, followed by three EGF-like domains. The C terminus is not conserved across the family, as Netrin-1, -3, -4 contain a C terminal netrin-like domain (NTR), whereas NetrinG1 and NetrinG2 contain sequences for GPI attachment.

Discovery of Netrin-1 receptors

DCC/Neogenin receptors

The first Netrin-1 receptor to be identified is DCC (deleted in colorectal carcinoma). DCC mediates chemoattraction in commissural axons (Keino-Masu et al, 1996), which will be discussed further under 'Axon guidance'. Neogenin, another member of the DCC family, is a receptor for Netrin-1 (Vielmetter et al, 1994) (Wilson & Key, 2006) and for Repulsive Guidance Molecule Family a (RGMa) (Rajagopalan et al, 2004) (**Figure-10**).

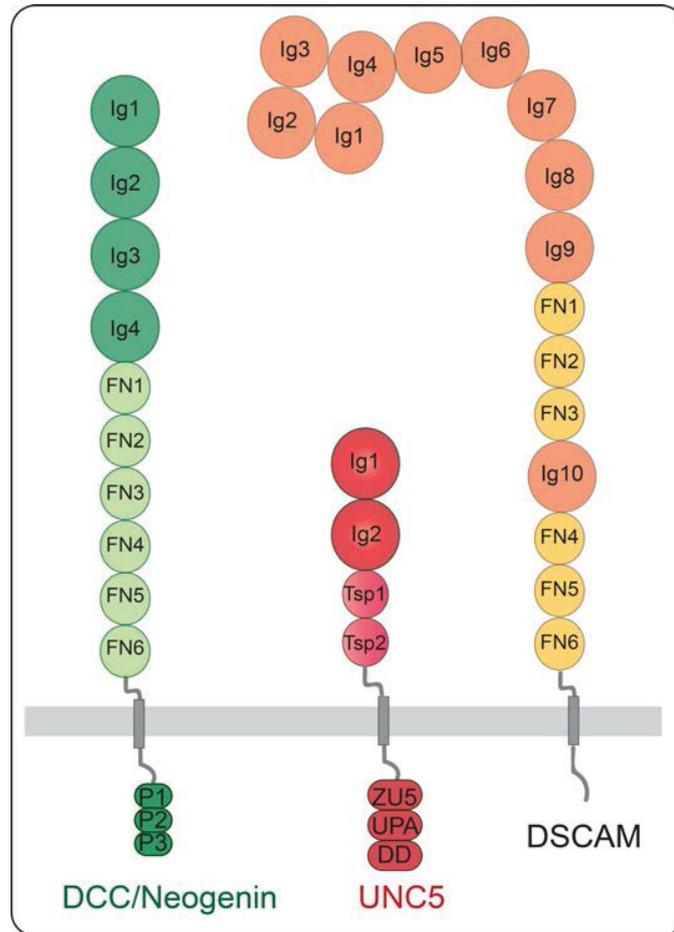


Figure-10: Main Netrin-1 Receptors Structures

DCC and Neogenin are composed of an extracellular domain consisting of four N-terminal Ig (immunoglobulin) C2-type domains (Ig1–4), and six FN (fibronectin) type III domains (FN1–6), followed by a single transmembrane helix, and an intracellular domain composed of P1-3 motifs. UNC5 family receptors (UNC5A–D) are composed of an extracellular domain consisting of two immunoglobulin (Ig1 and Ig2) and two thrombospondin type-I modules (Tsp1 and Tsp2) followed by an intercellular domain composed of zona occludens 5 (ZU5), UPA (or DB: DCC binding) and death domain (DD). DSCAM extracellular domain is composed of ten Ig domains and six FNIII repeats.

UNC5 family receptors

Netrins further interact with the repulsive receptor UNC5 (uncoordinated 5 homologs) family members (UNC5A–D). UNC5 was shown to switch the DCC-mediated attraction to the repulsion in response to Netrin-1 (Colamarino & Tessier-Lavigne, 1995) (**Figure-10**).

DSCAM

The Down syndrome cell adhesion molecule (DSCAM) is also a receptor for netrins (Andrews et al, 2008; Liu et al, 2009; Ly et al, 2008). DSCAM was shown to coordinate the Netrin-1-mediated cell attraction with DCC (Andrews et al, 2008) and Netrin-1-induced-cell repulsion with UNC5 receptors (Purohit et al, 2012) **(Figure-10)**.

Integrins

Integrins are a large family of transmembrane receptors that link the actin cytoskeleton to ECM proteins and are shown to bind Netrin-1 (Nikolopoulos & Giancotti, 2005). Netrin-1 binds to the $\alpha6\beta4$ and $\alpha3\beta1$ integrins, and this interaction is suggested to regulate epithelial cell adhesion and migration (Yebra et al, 2003).

APP

Netrin-1 binds to the β -amyloid precursor protein (APP) receptor and this interaction was shown to regulate Alzheimer's disease (Lourenco et al, 2009). APP also regulates commissural axon guidance by acting as a co-receptor for DCC (Rama et al, 2012).

A2b

The membrane-associated adenosine A2b receptor is also found as a Netrin-1 receptor functioning in a complex containing DCC/A2b/Netrin-1 to promote axonal outgrowth (Corset et al, 2000).

CD146

Recently, CD146 is shown to be a Netrin-1 receptor in promoting angiogenesis and vascular development (Tu et al, 2015).

Phenotypes of the mice homozygous mutant for Netrin-1 and its main receptors are depicted below (**Table-2**).

Knockout Mice	Lethality	References	Phenotype
Netrin-1	at 18.5 dpc	(Yung et al, 2015)	Defects in guidance of commissural axons, increased expression of DCC and NEO1
Netrin-1	at 14.5 dpc	(Bin et al, 2015)	Severe axon guidance defects, increased expression of DCC and NEO1
DCC (gene trap)	neonatal death at birth	(Fazeli et al, 1997)	Defects in brain morphogenesis and axon projections
NEO1 (gene trap)	no embryonic lethality	(Bae et al, 2009; Konig et al, 2012)	Decrease in myofiber size, Attenuated inflammatory response
UNC5A	no embryonic lethality	(Williams et al, 2006)	Decreased apoptosis, increased number of neurons in spinal cord
UNC5B	neonatal death at 12.5 dpc	(Lu et al, 2004)	Excessive blood vessel branching
UNC5C	neonatal death at birth	(Burgess et al, 2006)	Spinal motor axon development and trochlear axon guidance defects
UNC5D	no embryonic lethality	(Zhu et al, 2013)	Increase in neuron number

Table-2: The lethality and phenotypes of mice homozygous mutant for Netrin-1 and its main receptors.

Netrin-1 function

Netrin-1 function in the nervous system

Axon growth

Axon growth is the principal mechanism underlying the formation of neuronal networks. Each growing axon presents an amoeboid structure called 'growth cone' that has the capacity to interpret the 'guidance cues' to migrate along the correct path. The guidance cues found in axon microenvironment play a crucial role in axon growth and can be diffused as gradients (Kennedy et al, 2006). The four major axon cues are; netrins, semaphorins, ephrins and slits. In addition to guidance cues, there are also morphogenesis (WNT and Sonic Hedgehog (SHH) families) and cell adhesion molecules such as Fibronectin Leucine Rich Transmembrane Protein (FLRT) family that contribute to axon guidance (reviewed in (Seiradake et al, 2016)). The asymmetric distribution of receptor occupancy allows the polarization of the growth cone. The growth cone, migrating in a guidance cue gradient turn either toward increasing concentration of the cue (attraction) or away from the cue (repulsion) depending on the receptors present on the surface of migrating cone and through intracellular generation of second messengers such as Ca^{2+} and cyclic nucleotides. Moreover, the asymmetrically produced second messengers orchestrate multiple cellular systems including membrane trafficking, adhesion dynamics and cytoskeletal reorganization to execute bidirectional turning of the growth cone (Hedgecock et al, 1990; Hong et al, 1999; Hong et al, 2000; Long et al, 2004; Round & Stein, 2007; Togashi et al, 2008). According to the classical view, Netrin-1, one of the major member of the guidance cue family, is expressed by floor plate cells and a gradient of Netrin-1 protein is present in the embryonic spinal cord as commissural axons extend to ventral midline (Kennedy et al, 1994; Kennedy et al, 2006; Serafini et al, 1996; Tessier-Lavigne et al, 1988). However, a recent elegant study using conditional Netrin-1 knock-out models to delete Netrin-1 specifically either in floor plate or in ventricular zone, demonstrated that the attraction of

commissural axons is not mediated by a gradient of floor plate-derived Netrin-1. Moreover, Netrin-1 is expressed by neural stem cells localized in ventricular zone and primarily acts locally by promoting growth cone adhesion (Dominici et al, 2017). This recent finding strengthened the view that Netrin-1 modulates cell adhesion rather than being a diffusible cue for long-range chemoattraction in nervous system.

In contrast to other axon guidance cues, Netrin-1 has a very unique capacity for bifunctionality, meaning that it can guide opposed cellular responses according to the ligated receptor. In other words, bifunctional Netrin-1-mediated signaling may guide the axons in different directions due to the different receptor expression on the growth cone. The DCC receptor mediates chemoattractive response upon binding to Netrin-1, but also contributes to chemorepellent signaling together with UNC5 family receptors (Colavita & Culotti, 1998; Hong et al, 1999; Keino-Masu et al, 1996). The UNC5C receptor is required for Netrin-1 mediated-chemorepulsive effect (Przyborski et al, 1998). Moreover, in *Drosophila*, ectopic expression of UNC5 mediates short-range axon repulsion, whereas long-range repulsion also requires Frazzled/DCC (Keleman & Dickson, 2001). Altogether these studies identified that Netrin-1 is a bifunctional axon guidance cue by acting as an attractive or as a repulsive cue for axonal growth **(Figure-11)**.

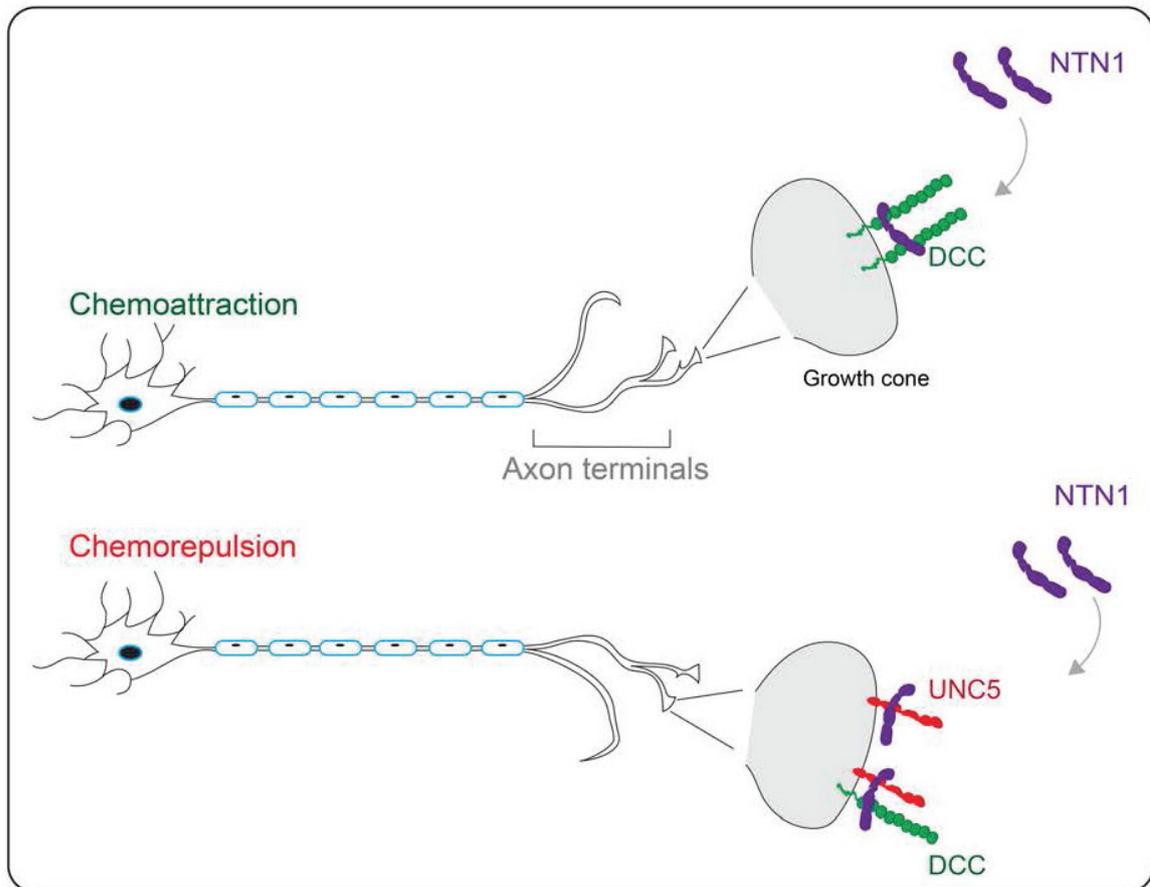


Figure-11: Model for Netrin-1 bifunctionality in axon growth

In response to Netrin-1, homodimerization of the DCC receptors mediates chemoattraction. In contrast, UNC5 receptors alone or UNC5-DCC heterodimers mediate chemorepulsion in response to Netrin-1.

Neuronal cell migration

Netrin-1 was also shown to act as an attractive or repulsive cue for distinct migrating neurons during cerebellar system development (Alcantara et al, 2000). Netrin-1-mediated neuronal migration and axonal guidance require phosphorylation and activation of microtubule-associated protein MAP1B. The activation of MAP1B depends on the kinases GSK3 and CDK5 (Del Rio et al, 2004). Netrin-1 also acts as a key repellent factor in neural stem cell migration in the injured adult nervous system (Petit et al, 2007). Besides axon growth and

neuronal migration, Netrin-1 also plays roles in related neurological processes such as axon branching, innervation, synaptogenesis and oligodendroglial development and maturation (reviewed in (Lai Wing Sun et al, 2011)).

Netrin-1 outside the nervous system: The one with “pleiotropic functions”

Several studies identified Netrin-1 and its receptors in various cell types and in various cellular processes beyond the neuronal system indicating that Netrin-1 is a protein with pleiotropic functions. For example, Netrin-1 is expressed in otic epithelium and important for inner ear development. Netrin-1 plays a role in lung epithelial branching morphogenesis through inhibiting phospho-ERK-1/2 activity (Liu et al, 2004). During mammary gland morphogenesis, Netrin-1 and its receptor Neogenin interaction stabilizes multipotent progenitor cap cells and provide an adhesive function (Srinivasan et al, 2003). In the pancreas, Netrin-1 stimulates the migration of human pancreatic duct and porcine islet precursor cells and through interacting with $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins that mediates cell adhesion and migration of putative pancreatic progenitor cells (De Breuck et al, 2003; Yebra et al, 2003).

Netrin-1 functions as a guidance cue also in angiogenesis. Netrin-1 limits endothelial cell migration and blood vessel branching when UNC5B is present (Lu et al, 2004) (Larrivee et al, 2007). However, in contrast to such finding, other studies show that Netrin-1 promotes angiogenesis (Wilson et al, 2006) (Park et al, 2004). Recently, CD146 has been identified as a Netrin-1 receptor that promotes angiogenesis and vascular development (Tu et al, 2015). These results suggest that Netrin-1 might harbor bifunctional activity in different process such as angiogenesis. However, the receptor distributions in differing angiogenic response are not well characterized.

Recent findings also attribute immunomodulatory functions to Netrin-1. For example, Netrin-1 inhibits leukocyte migration (Ly et al, 2005). Netrin-1 dampens

inflammation in various tissues and context such as: hypoxia-induced, injury-induced inflammation in lung and kidney (Mutz et al, 2010; Rosenberger et al, 2009; Tadavadi et al, 2010). On the other hand, Netrin-1, via its receptor UNC5B, is shown as an inflammatory factor that inhibits macrophage migration in adipose tissue during obesity, thereby promoting chronic inflammation and insulin resistance (Ramkhalawon et al, 2014). These results suggest that Netrin-1 might also harbor bifunctional properties in regulation of inflammation depending on the context and possibly by differential receptor expression.

Netrin-1 also contributes to tissue homeostasis, liver regeneration (Schlegel et al, 2016), nerve regeneration (Dun & Parkinson, 2017), protects bone erosion in an autoimmune arthritis model (Maruyama et al, 2016) and promotes mesenchymal stem cell revascularization of limb ischemia (Ke et al, 2016). These findings, together with the new discovery indicating that Netrin-1 is expressed by neural stem cells (Dominici et al, 2017), suggest a role for Netrin-1 in different stem cell models. However, dual function of Netrin-1 in stem cell models is not described till today. It would be interesting to screen different stem cell models to further identify if Netrin-1 is a common regulator of stemness.

Netrin-1 outside the nervous system: The ligand of 'Dependence Receptors'

Netrin-1 receptors DCC, Neogenin and UNC5 family members (UNC5A-D) have also been shown to belong to the dependence receptor family. Dependence Receptors are transmembrane receptors with dual functions. These receptors, similar to the classical receptors, induce "positive signals" such as axon guidance, proliferation, migration and cell survival when they are bound to their ligands. However, when they are unbound by their ligands, these receptors transduce "negative signals" that actively induce apoptosis. These receptors are implicated in nervous system development, cancer progression and angiogenesis (Castets et al, 2012; Castets et al,

2009; Delloye-Bourgeois et al, 2009a; Fitamant et al, 2008; Goldschneider & Mehlen, 2010; Goldschneider et al, 2008; Guenebeaud et al, 2010) (**Figure-12**).

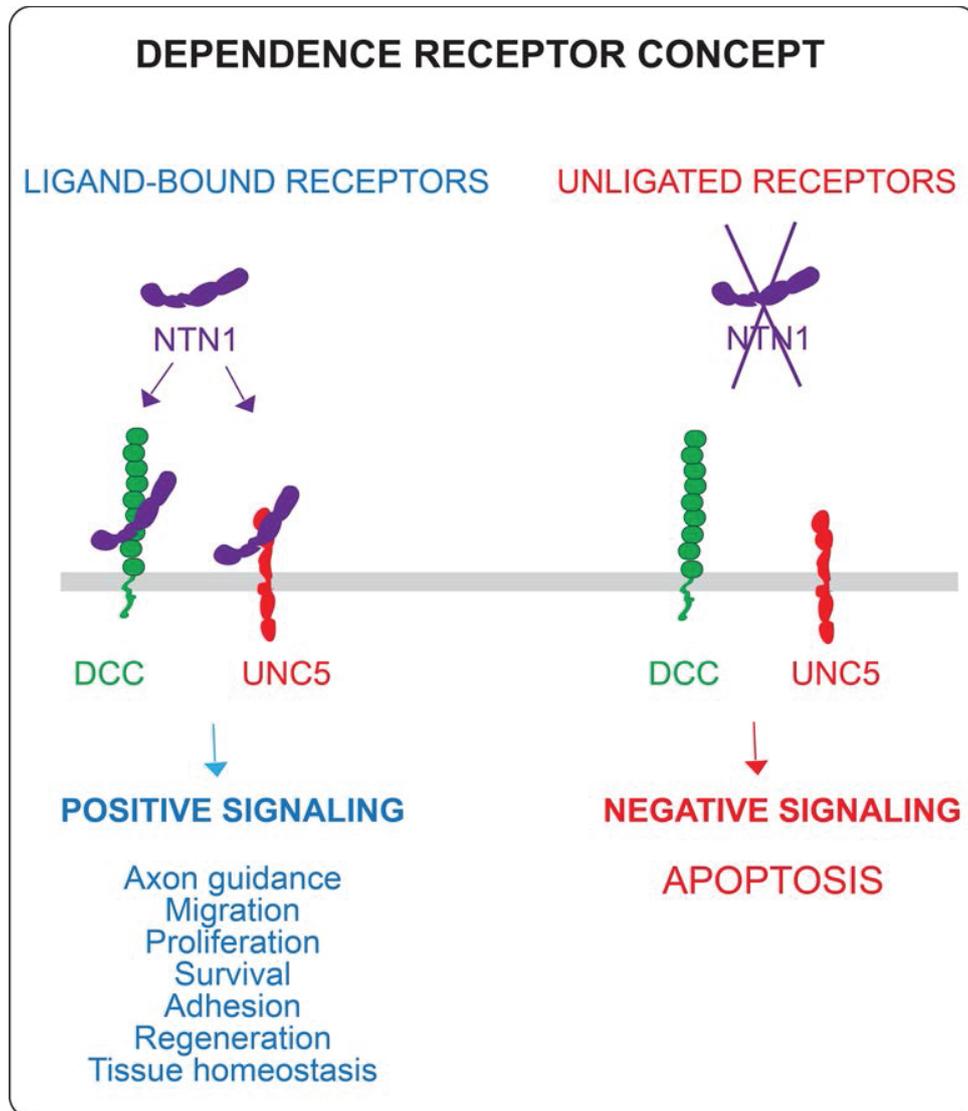


Figure-12: Model for “Dependence Receptor” concept

In the presence of Netrin-1, DCC/Neogenin and UNC5 family receptors induce positive signaling. In the absence of Netrin-1, DCC/Neogenin and UNC5 family receptors actively induce apoptosis.

During embryonic development, Netrin-1 acts as survival factor for olfactory neurons by inhibiting apoptosis upon binding to DCC and UNC5 receptors (Llambi et

al, 2001). Moreover, Netrin-1 is a survival factor for spinal cord commissural neurons (Furne et al, 2008).

During tumoral progression, dependence receptors are considered to be tumor suppressors due to their ability to promote cell death when they are disengaged from their ligand. However, tumor cells can escape from dependence receptor-induced cell death by either the loss of dependence receptors apoptotic activity or by gain of ligand expression (Goldschneider & Mehlen, 2010).

Supporting the view of the ligand expression amplification, Netrin-1 has been shown to be upregulated in metastatic breast, lung, ovary cancers, in pancreatic cancer and in inflammatory-associated-colorectal cancer and in neuroblastoma (Delloye-Bourgeois et al, 2009a; Delloye-Bourgeois et al, 2009b; Dumartin et al, 2010; Fitamant et al, 2008; Papanastasiou et al, 2011; Paradisi et al, 2009).

The tumor suppressor DCC expression is lost in colon, breast, endometrial ovarian, prostate, gastric, esophageal, breast, testicular cancers and neuroblastoma (Mehlen & Fearon, 2004) (Fearon et al, 1990). Loss of DCC expression is often associated with poor prognosis and advanced cancer or metastasis (Saito et al, 1999; Shibata et al, 1996) suggesting a role for DCC in cancer progression rather than in cancer initiation. The tumor suppressor activity of DCC is engaged by caspase cleavage of DCC at Asp 1290 (Mehlen et al, 1998). The role of tumor suppressor activity of the DCC in cancer progression was revealed using mice carrying a mutation on the DCC caspase cleavage site (D1290N). In this study, the scientist crossed the APC^{+/1638N} mutant background that is predisposed to form adenocarcinomas but not metastases, with the DCC mice carrying the D1290N mutation. They observed increased adenocarcinoma formation with serosal invasion and micrometastases due to the loss of DCC apoptotic activity (Castets et al, 2011). This finding strengthens the view for the tumor suppressor role of DCC by engaging an apoptotic process in cancer progression and metastasis.

UNC5 receptor family is also lost in colorectal, breast, ovary, uterus, stomach, lung or kidney cancers (Thiebault et al, 2003). The UNC5C receptor expression is decreased in a large fraction of human colorectal cancers through promoter methylation. Moreover, in mice, inactivation of UNC5C is associated with increased intestinal tumor progression and a decrease in tumor cell apoptosis (Bernet et al, 2007).

The dependence receptors as therapeutic targets

Cancer cells could escape from tumoral clearance by up-regulating ligand expression or by loss of dependence receptor apoptotic activity. The dependence receptors could be down-regulated due to promoter methylation, could carry mutations altering their apoptotic activity, or, an apoptotic partner protein could be lost (Delloye-Bourgeois et al, 2009a; Fitamant et al, 2008; Grandin et al, 2016b; Mille et al, 2009). For this purpose, targeting the cancer cells with Netrin-1 interference and combining it with chemotherapeutic agents (Paradisi et al, 2013) or with DNA methylation inhibitors (Grandin et al, 2016a) may be promising approaches to control cancer cell progression and to limit tumor cell escape from therapy.

Beside its survival function in tumorigenesis, Netrin-1 guides pleiotropic functions for survival of other tissues. Netrin-1 was shown to be a survival factor in angiogenesis. In this case, the dependence receptor UNC5B controls the survival of endothelial cells and promotes angiogenesis (Castets et al, 2009). Furthermore, Netrin-1 is expressed by different stem cells. It would be interesting to identify further Netrin-1 and its receptors function to regulate stem cell hemostasis. In this context, Netrin-1 and its receptors may represent viable targets in the control of stem cell hemostasis.

Structure of Netrin-1 and its receptors

Structure of netrins

All netrins are composed of approximately 600 amino acids and are related to laminin family proteins (Yurchenco & Wadsworth, 2004). All netrins are also composed of an N-terminal laminin VI domain (Lam or LN), followed by three laminin-type epidermal growth factor (LE) repeats (EGF1, EGF2 and EGF3 or LE1, LE2 and LE3) (Serafini et al, 1994; Wang et al, 1999). However the C terminus is not conserved across the family. Netrin-1, -3, and -4 contain a C-terminal netrin-like domain, whereas NetrinG1 and NetrinG2 contain sequences for GPI attachment to membrane and are present only in vertebrates (Nakashiba et al, 2002) (Miyashita et al, 2005) **(Figure-13)**. Among netrins, Netrin-1 and is the most studied protein.

Structure of Netrin-1 receptors

DCC/Neogenin receptors

The DCC extracellular domain consists of four N-terminal Ig C2-type domains (Ig1–4), and six FN type III domains (FN1–6), followed by a single transmembrane helix, and an intracellular domain composed of P1-3 motifs (Cho et al, 1994) (Kolodziej et al, 1996) **(Figure-13)**. The receptor Neogenin is another member of DCC family that is structurally related to DCC. It also binds Netrin-1 (Vielmetter et al, 1994) (Wilson & Key, 2006) and another repulsive guidance molecule RGMa (Rajagopalan et al, 2004). Recently, crystallography revealed the structural mechanisms of DCC/Neogenin-Netrin-1 interaction. Two recent publications showed the interaction between DCC/Neogenin FN5 domain and the third EGF domain (LE3) of Netrin-1 (Finci et al, 2014b) (Xu et al, 2014). Additionally, the DCC/Neogenin FN4 domain and the Netrin-1 Laminin (LN) domain are identified to form complexes (Xu

et al, 2014) **(Figure-13)**. Negatively charged sulfated glycans of the heparan sulfate family also bind to Netrin-1 and its receptor DCC (Bennett et al, 1997; Serafini et al, 1994). Recent crystal structure-based work also identified that, when heparan sulfate HP1 is bound to netrin-1, a second DCC molecule is recruited to binding site 2, and DCC homodimers form a signaling complex, leading to attraction of the growth cone. However, when heparan sulfate HP2 binds to netrin-1, UNC5A is recruited to the EGF-1/EGF-2 domains, DCC and UNC5A heterodimers form a signaling complex, leading to repulsion of the growth cone (Finci et al, 2014a). These recent findings also elucidate the importance of differential structural protein complexes and their impact on opposed cellular responses such as chemoattraction and chemorepulsion.

UNC5 family receptors

The UNC5 receptors are transmembrane proteins with an extracellular domain consisting of two immunoglobulin (Ig1 and Ig2) and two thrombospondin type-I modules extracellular (Tsp1 and Tsp2) followed by 3 intercellular domains, namely zona occludes 5 (ZU5), UPA (or DB: DCC binding) and death domain (DD) (Hong et al, 1999; Leonardo et al, 1997; Wang et al, 2009) **(Figure-13)**. X-Ray crystallography reveals the interaction between Netrin-1 EGF2 (LE2) domain and the Ig domains, especially Ig1, of UNC5B (Grandin et al, 2016b). It was shown that Netrin-1 is capable of switching from a chemo-attractant to a chemo-repulsive guidance cue when UNC5 homologues are present on the cell surface of the growth cone, in combination with DCC (Hong et al, 1999). This result identified that the composition of receptors found in growth cone membrane is an important regulator of growth cone turning in response to Netrin-1. *In vitro* axon guidance assays revealed several necessary interactions, such as DCC-FN5 binding to Netrin-EGF3 that is required for DCC-mediated attraction and for UNC5A/DCC-mediated repulsion. DCC-FN5-FN6 linker binding to Netrin-EGF2 also contributes to DCC-mediated attraction, but not to UNC5A/DCC-mediated repulsion (Finci et al, 2014a).

These results suggest that Netrin-1 EGF2 is the key domain for receptor binding **(Figure-13)**. Moreover, there is a strong concentration of positively charged residues in the binding site consisting of DCC-FN5-FN6 /Netrin-EGF2 (on both DCC and Netrin-1). The positively charged patches are bridged and neutralized by a cluster of anions at the interface (Finci et al, 2014a). Previously, the FN5 domain of DCC was identified to both bind to negatively charged molecules heparin or heparan sulfates as well as a netrin-1 blocking monoclonal antibody (Bennett et al, 1997). Further analysis indicated the formation of a ternary DCC/heparin/netrin-1 complex in this region (Geisbrecht et al, 2003). Furthermore, it was previously observed that the two immunoglobulin domains of UNC5 receptors bind netrin-1 through heparin (Geisbrecht et al, 2003). The crystal structure-based studies show that Netrin-1 associates with a heparan sulfate molecule that is selective for DCC (HP1) or UNC5 (HP2) and netrin-1 signaling-mediated co-receptor selection is tuned by heparan sulfates and other environmental factors, leading to alternate cues, and this is fundamental to the netrin-based guidance code (Geisbrecht et al, 2003). These results indicate that differential heparan sulfate-binding to netrin-receptor complexes is important for Netrin-1 bi-functionality in guiding both chemoattractive and chemorepulsive responses.

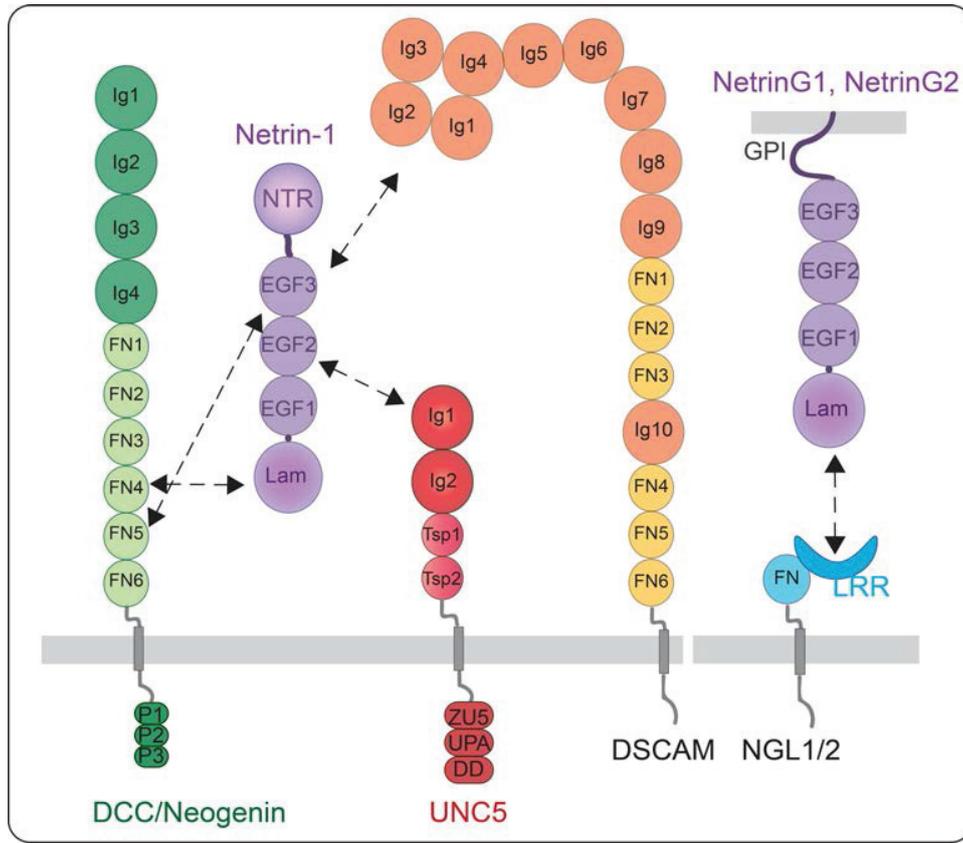


Figure-13: Main Netrin-1 Receptors and their interaction domains with Netrin-1

Netrin-1 Lam (LN) domain interacts with DCC/Neogenin FN4 domain. Netrin-1 EGF3 domain interacts with DCC/Neogenin FN5 domain. Netrin-1 EGF2 domain interacts with UNC5 receptors Ig1 domain. Netrin-1 EGF3 domain interacts with DSCAM Ig loops. Membrane-anchored NetrinG1 and NetrinG2 Lam (LN) domain binds to concave surface of the LRR domains of NetrinG Ligand (NGL) (Seiradake et al, 2011).

DSCAM receptor

The DSCAM extracellular domain is composed of ten Ig domains and six FNIII repeats (Purohit et al, 2012) and Netrin-1 is proposed to bind to the Ig loops (Ly et al, 2008).

Netrin-1 signaling

Netrin-1 signaling in chemoattraction

In axon growth cone, signal transduction molecules link the activation of axon guidance receptors to the reorganization of the actin cytoskeleton through activation of signaling pathways (reviewed in (Huber et al, 2003)). Netrin-1 can activate multiple downstream signal transduction molecules such as SRC family kinases (SFKs) and Rho GTPase family members that regulate cytoskeletal dynamics in growth cone (Huber et al, 2003).

The intracellular domain of DCC is constitutively bound to Focal Adhesion Kinase (FAK; PTK2) and to the adaptor protein NCK1 that serves as scaffold (Li et al, 2004; Li et al, 2002a). Upon Netrin-1 binding, DCC is dimerized leading to activation of FAK and SRC, and tyrosine phosphorylation of the DCC intracellular domain (Li et al, 2004), and activation of FYN (Li et al, 2004; Meriane et al, 2004) that activates Rho GTPases RAC1 and CDC42 (Li et al, 2002b) and inhibits Rho GTPase RHOA (Moore et al, 2008). The adaptor protein PAK1 links NCK1 to RAC1 and CDC42 (Bagrodia & Cerione, 1999). Moreover, Enabled/vasodilator-stimulated phosphoprotein (ENA/VASP) and neuronal Wiskott-Aldrich syndrome protein (N-WASP) are downstream regulators of DCC activated CDC42 and they remodel actin network in response to guidance cues (Lebrand et al, 2004; Shekarabi et al, 2005).

The signaling cascade initiated upon Netrin-1 binding to DCC, also involves phosphorylation of the extracellular regulated kinases 1 and 2 (ERK1 and ERK2) and the activation of mitogen-activated protein kinase (MAPK) cascade and affects protein synthesis (Forcet et al, 2002; Ma et al, 2010), promotes the synthesis of the phosphoinositide phosphatidylinositol (4,5) bisphosphate (PIP₂) (Xie et al, 2005), which is phosphorylated by phosphatidylinositol-3 kinase (PI3K) and results in phosphatidylinositol (3,4,5) trisphosphate (PIP₃) production that can activate pAKT/mTOR pathway (reviewed in (Lai Wing Sun et al, 2011)) (Xie et al, 2005).

Netrin-1 also induces PIP2 hydrolysis by phospholipase C γ (PLC γ) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), which activates protein kinase C (PKC) that mediates cytoskeletal rearrangements and translational control, and stimulate the release of Ca²⁺ from intracellular stores that is required for cone steering (Hong et al, 2000; Larsson, 2006; Ming et al, 1999). These results indicate that Netrin-1/DCC interaction could affect several signaling pathways regulating various cellular responses, which regulate translation, calcium flux and cytoskeletal remodeling (**Figure-14**). It could be interesting to investigate if Netrin-1/DCC interaction triggers different signaling pathways in different cellular contexts.

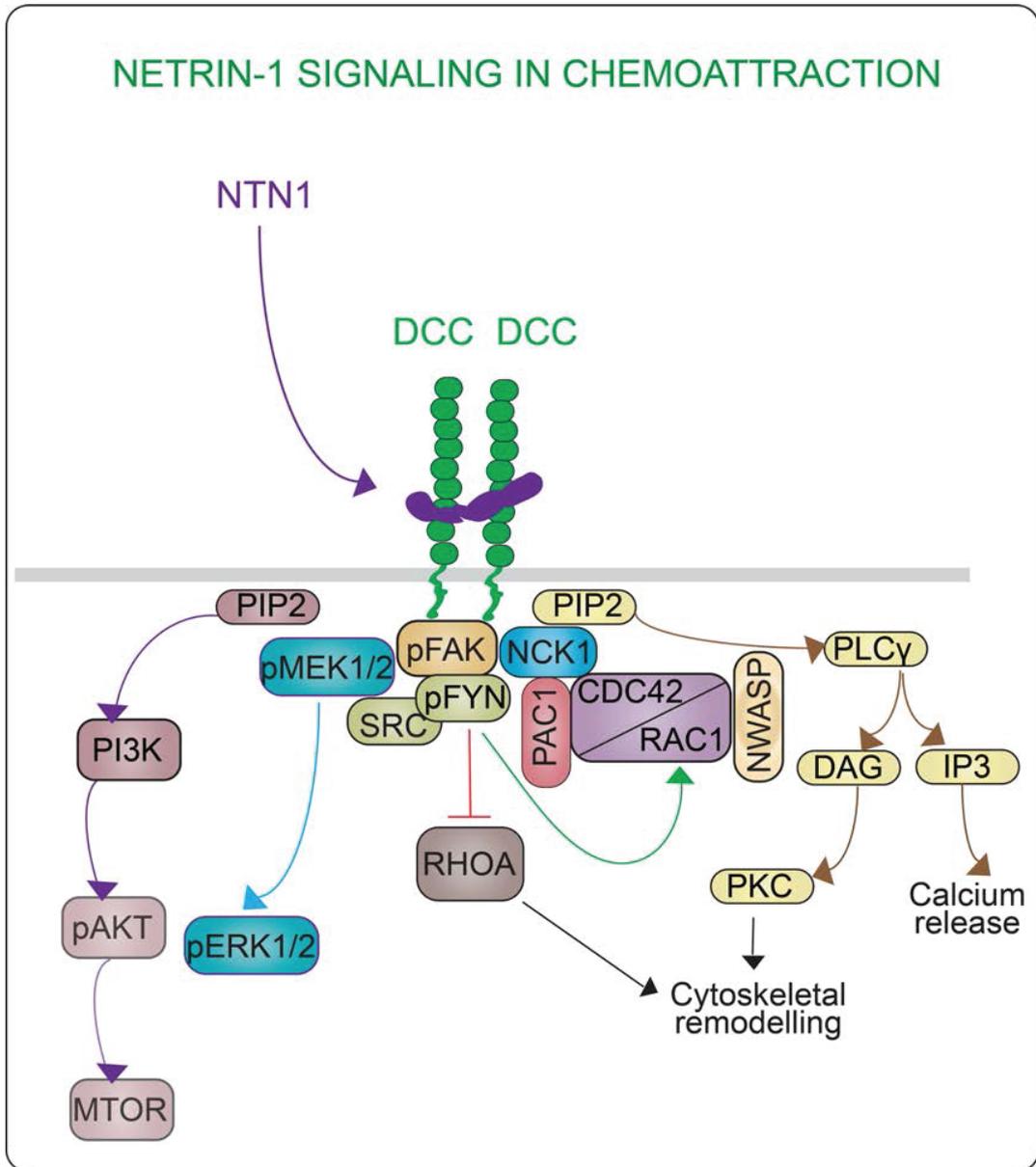


Figure-14: Model for Netrin-1 signaling in chemoattraction

Netrin-1 binding to DCC homodimers induces chemoattraction via activation of FAK, SRC, FYN, Rho GTPase RAC1, CDC42, N-WASP (modulators of cytoskeleton), MAPK cascade, PI3K pathway, PKC (modulator of cytoskeleton) and IP3 generation (calcium release).

Netrin-1 signaling in chemorepulsion

In axon growth, there are two main chemorepulsive responses: the long-range repellent response mediated by UNC5/DCC heterodimers and the short-range repellent response mediated by UNC5 receptors in the absence of DCC (Hong et al, 1999; Keleman & Dickson, 2001). Netrin-1 induces the phosphorylation of UNC5 (on Y482) in a DCC-dependent manner, through the actions of SRC and FAK (Killeen et al, 2002; Li et al, 2006) and induces the tyrosine phosphatase SHP2 (PTPN11) to bind to UNC5 (Tong et al, 2001). The PAK family member MAX-2 and the adaptor protein MAX-1 are also modulators of UNC-5- mediated axon repulsion (Huang et al, 2002; Lucanic et al, 2006) (**Figure-15**).

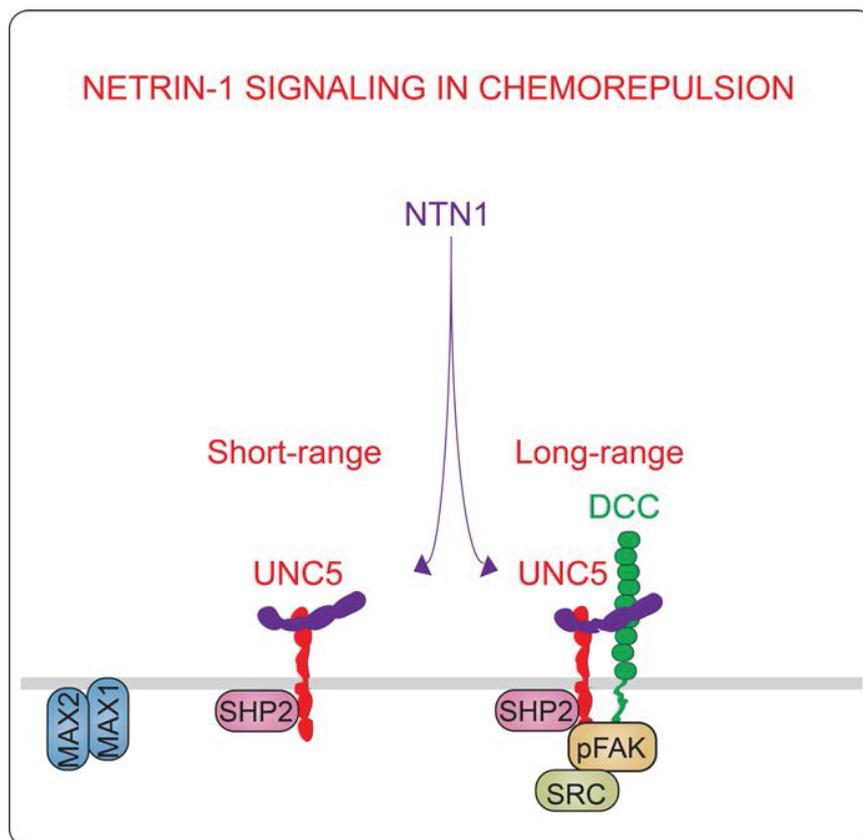


Figure-15: Model for Netrin-1 signaling in chemorepulsion

Netrin-1 binding to UNC5 homodimers induces short-range chemorepulsion and involves Shp2, Max1, Max2. Netrin-1 binding to UNC5/DCC heterodimers induces long-range chemorepulsion and involves recruitment of Shp2, activation of Fak and SRC.

Netrin-1 signaling in cell adhesion

Netrin-1 and DCC were shown to be regulators of cell adhesion. (Moore et al, 2008). Furthermore, during chemoattraction, DCC functions as a transmembrane bridge that links extracellular Netrin-1 to the actin cytoskeleton, forming the core of a protein complex that directs the reorganization of F-actin (Lai Wing Sun et al, 2011). Outside of the nervous system, Netrin-1, -3 and -4, DCC and Neogenin are regulators of cellular adhesion in epithelial morphogenesis in the mammary gland, pancreas, lung and lymphatic vasculature (Hebrok & Reichardt, 2004; Larrieu-Lahargue et al, 2010; Liu et al, 2004; Slorach & Werb, 2003; Srinivasan et al, 2003; Yebra et al, 2003). The intracellular domain of DCC also binds to ezrin and merlin (neurofibromin 2) to regulate cell adhesions (Martin et al, 2006). UNC5B and UNC5D have recently been shown to bind with high affinity to the ectodomain (ECD) of Fibronectin and Leucine-Rich Transmembrane protein-3 (FLRT3) and -2 (FLRT2) respectively. The transmembrane proteins, FLRTs, are regulators of fibroblast growth factor (FGF) mediated signaling and are thought to act cell autonomously either as a co-receptor or as cell adhesion molecules in early embryogenesis (Bottcher et al, 2004; Karaulanov et al, 2006; Seiradake et al, 2014; Yamagishi et al, 2011). The UNC5B/FLRT3 complex regulates cell adhesion during early embryogenesis through the Rho small GTPase via controlling cell surface levels of cadherin through a dynamin-dependent endocytosis pathway (Ogata et al, 2007). Moreover, as FLRT2/UNC5D signaling modulates cortical neuron migration (Yamagishi et al, 2011), these findings suggest that Netrin-1 signaling and UNC5 interaction with FLRTs is an important modulator of cell adhesion. It would be also interesting to investigate whether UNC5 receptors interaction with FLRTs could modulate FGF signaling. After all, Netrin-1 is a survival factor in tumoral progression, and Cell Adhesion Molecule (CAM) and cadherins are important regulators of cancer progression (Okegawa et al, 2004). Thus Netrin-1 signaling and

FLRTs may regulate cellular adhesion, and could have an impact in control of tumorigenesis.

Protein-based screens could be used to identify if Netrin-1 receptors interact with other cellular adhesion proteins and/ or adaptor proteins and kinases to better understand Netrin-1 signaling function in regulation of cellular adhesion.

Control of Netrin-1 signaling

Receptor trafficking, cAMP and calcium in Netrin-1 signaling modulation

The generation of second messenger, cyclic adenosine monophosphate (cAMP) is one of the major drivers of growth cone guidance. cAMP can activate protein kinase A (PKA), which in turn regulates Rho GTPase activation (Lang et al, 1996) and Enabled/vasodilator-stimulated phosphoprotein (ENA/VASP) function (Krause et al, 2003), to regulate cytoskeletal dynamics downstream of Netrin-1 signaling (Lebrand et al, 2004; Moore et al, 2008; Shekarabi et al, 2005).

Rho signaling reduces axonal chemoattraction to Netrin -1 by limiting the amount of plasma membrane DCC at the growth cone, and Netrin-1-mediated inhibition of RhoA facilitates chemoattraction to Netrin -1(Moore et al, 2008). Moreover, PKA activation promotes the relocation of DCC from an intracellular vesicular pool to the plasma membrane to regulate axonal extension (Bouchard et al, 2004). These results indicate both PKA and RhoA control receptor trafficking in growth cone. On the other hand, the activation of PKC and the recruitment of Protein Interacting with C kinase 1 (PICK1) to the UNC5A intracellular domain trigger endocytosis of UNC5A but not of DCC. The endocytosis of UNC5A converts the Netrin-1-stimulated chemorepulsion to attraction (Bartoe et al, 2006; Williams et al, 2003). These

findings identify the regulation of receptor trafficking as a key determinant of the Netrin-1-mediated responses.

Decreasing cytoplasmic Ca²⁺ levels, by blocking Ca²⁺ release from intracellular stores or by inhibiting its influx through Ca²⁺ channels, may also convert neuron responses to Netrin-1 from attraction to repulsion (Hong et al, 2000). This requires calcium-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) phosphatase. High local Ca²⁺ concentrations favor CaMKII-induced-attraction and moderate levels of Ca²⁺ activates CaN phosphatase mediated-repulsion. Moreover, cAMP pathway negatively regulates CaN phosphatase to inhibit repulsion(Wen et al, 2004). Furthermore a higher intracellular ratio of cAMP to cGMP promotes the attraction of neuron growth cones to Netrin-1 by activating Ca²⁺ entry through L-type calcium channels (LCCs), whereas a lower ratio of cAMP to cGMP results in decreased Ca²⁺ influx and Netrin -1-mediated repulsion (Nishiyama et al, 2003). These results suggest that, cAMP and cGMP signaling, modulation of Ca²⁺ channel activity in growth cones and receptor trafficking are early membrane-associated events of Netrin-1 signaling (**Figure-16**).

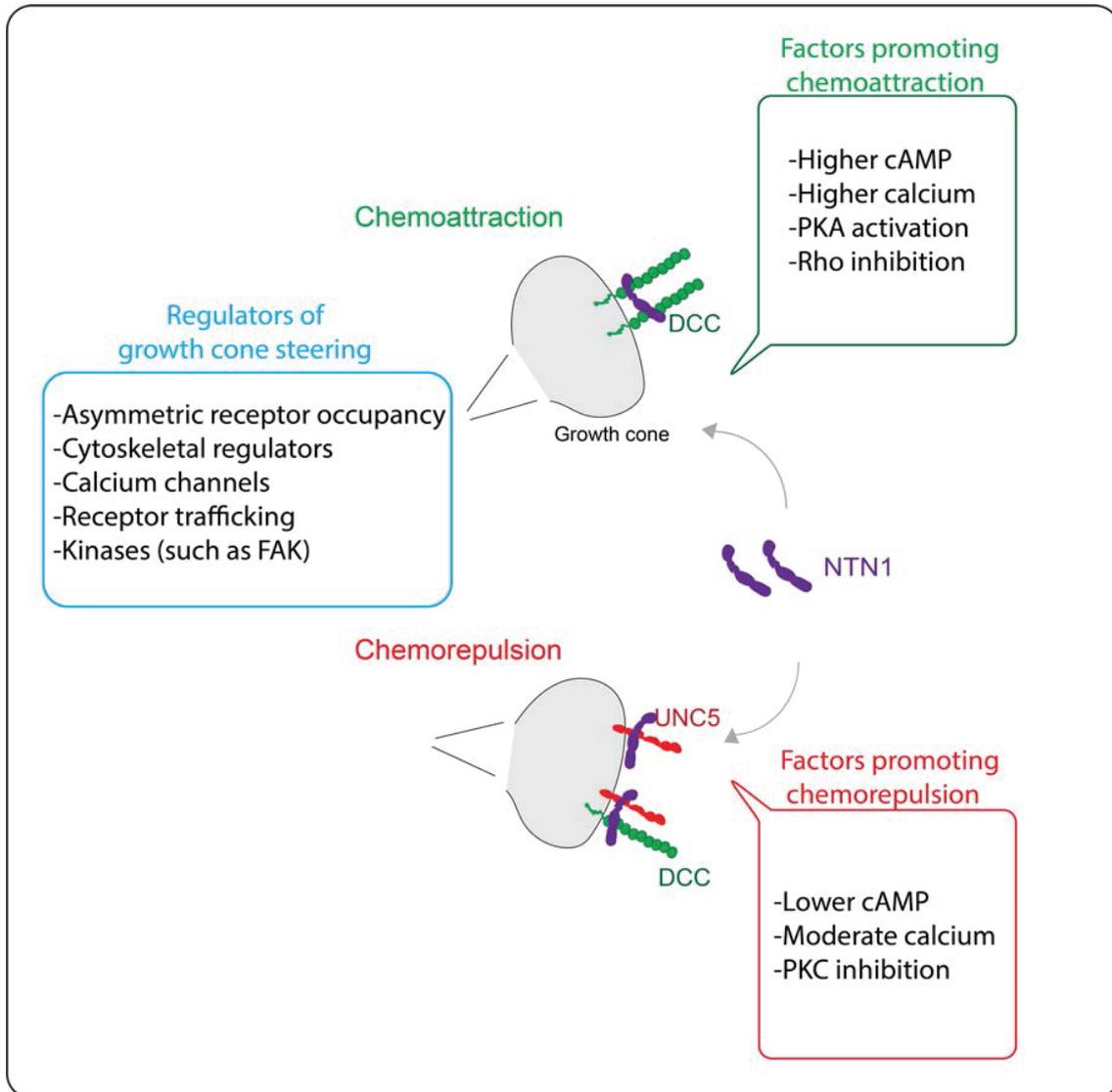


Figure-16: Model for mediators affecting growth cone steering in response to Netrin-1
 Calcium, cAMP, receptor trafficking, kinases activity, cytoskeletal arrangements are modulators of chemoattraction and chemorepulsion.

Negative signaling induced by the Dependence Receptors

The receptors of Netrin-1: UNC5s, DCC/Neogenin, belong to "Dependence Receptor" family. These receptors are able to actively trigger cell death in the absence of their ligand.

In the absence of netrin-1, caspase-9 is recruited to DCC, and drives caspase-3 activation to initiate cell death (Forcet et al, 2001). DIP13 α (DCC-interacting protein 13- α), a protein identified as an interactor of DCC ADD, is critical for DCC-induced cell death (Liu et al, 2002). In addition, palmitoylation and lipid raft localization were reported to be a prerequisite for DCC proapoptotic activity (Furne et al, 2006). DCC presence in lipid rafts is required for caspase-9/DCC interaction, suggesting that this caspase-activating complex occurs in lipid rafts. Like DCC, UNC5s also require lipid raft association to induce cell death (Maisse et al, 2008).

The formation of a caspase-activating complex does not seem to be the only mechanism used by DRs to trigger apoptosis. UNC5A induces apoptosis by interacting with NRAGE (neurotrophin receptor-interacting MAGE homolog) through its ZU5 domain (Williams et al, 2003). NRAGE possibly transduces UNC5A-induced apoptosis through degradation of the caspase inhibitor X-linked inhibitor of apoptosis (XIAP), and on the other hand, through activation of the proapoptotic c-Jun N-terminal kinase (JNK) signaling pathway.

UNC5B triggers apoptosis mainly by recruiting the serine/threonine Death-Associated Protein Kinase (DAPK) (Llambi et al, 2005) that is activated by PP2A (Guenebeaud et al, 2010). Moreover, DAPK is already known to trigger cell death through p53-dependent and -independent mechanisms and phosphorylation of the myosin light chain by DAPK leads to membrane blebbing, a hallmark of programmed cell death (Gozuacik & Kimchi, 2006) . Interestingly, Netrin-1 induces interaction of UNC5B with the brain-specific GTPase PIKE-L and this interaction prevents UNC5B's pro-apoptotic activity and enhances neuronal survival (Tang et al, 2008).

Neogenin also interacts with DAPK and requires DAPK for apoptosis induction (Fujita et al, 2008). Neogenin is cleaved by γ -secretase or caspase. The ligand γ -secretase cleavage appears to promote nuclear signaling speculated to be

antiapoptotic, while the caspase cleavage promotes the Neogenin proapoptotic activity (Goldschneider et al, 2008; Matsunaga et al, 2004). Moreover, the engagement toward Neogenin-mediated apoptosis is associated not only with apoptosis promotion by the exposure of the Neogenin proapoptotic domain but also with the inhibition of Neogenin intracellular domain (NeICD)-mediated transcription. It would be interesting to identify if unbound dependence receptors, beside their active apoptotic functions, could induce different cellular responses such as differentiation in control of tumorigenesis or in different physiological or pathological contexts.

RESULTS

During my thesis, I initially worked in a project leading to the identification of Netrin-1 as a regulator of reprogramming and pluripotency. Using a Chip-seq based screen centered on genomic regions which are differentially bound by OSKM in somatic and pluripotent cells, we identified Netrin-1, and its receptors DCC and UNC5 family members, as new candidate regulators of reprogramming. During this reprogramming, DCC-triggered-cell death led to poor efficiency in the iPSC generation process. The iPSC generation techniques were improved by the simple and noninvasive treatment with recombinant Netrin-1, in both mouse and human cells. Furthermore, similarly, exogenous Netrin-1 expression improved mESC self-renewal by blocking UNC5B-induced apoptosis. This work is published in the journal *Nature Communications*.

During my thesis, I also determined that Netrin-1 signaling is dynamically regulated in distinct pluripotency states and in lineage commitment. Netrin-1 signaling was found to stimulate remarkably opposite effects during pluripotency and lineage commitment. This bifunctionality is reminiscent of its capacity to mediate opposed cellular responses during nervous system development. In PSCs, Netrin-1 signals via Neogenin and UNC5B receptors. For the naïve PSCs, Netrin-1 enhances self-renewal, delays differentiation, increases WNT sensitivity and decreases responsiveness to FGF, which is dependent upon Netrin-1-induced UNC5B internalization and subsequent degradation. Once a cell enters a state of primed pluripotency, UNC5B expression is strongly elevated. In this case, Netrin-1 stimulates differentiation towards lineage commitment by potentiating FGF signaling. In this work, I characterized the unique and unexpected conservation of bifunctionality in Netrin-1 signaling during early embryogenesis, acting to cue both naïve pluripotency and lineage commitment. This work is presented in the form of PDF and is in submission.

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Netrin-1 regulates somatic cell reprogramming and pluripotency maintenance

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The generation of induced pluripotent stem (iPS) cells holds great promise in regenerative medicine. The use of the transcription factors Oct4, Sox2, Klf4 and c-Myc for reprogramming is extensively documented, but comparatively little is known about soluble molecules promoting reprogramming. Here we identify the secreted cue Netrin-1 and its receptor DCC, described for their respective survival/death functions in normal and oncogenic contexts, as reprogramming modulators. In various somatic cells, we found that reprogramming is accompanied by a transient transcriptional repression of Netrin-1 mediated by an Mbd3/Mta1/Chd4-containing NuRD complex. Mechanistically, Netrin-1 imbalance induces apoptosis mediated by the receptor DCC in a p53-independent manner. Correction of the Netrin-1/DCC equilibrium constrains apoptosis and improves reprogramming efficiency. Our work also sheds light on Netrin-1's function in protecting embryonic stem cells from apoptosis mediated by its receptor UNC5b, and shows that the treatment with recombinant Netrin-1 improves the generation of mouse and human iPS cells.

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Somatic cell reprogramming to pluripotency involves epigenetic modifications, changes in gene expression, protein degradation and protein synthesis. Reprogramming resets differentiated cells to a pluripotent state and can be achieved by nuclear transfer, cell fusion or transduction of certain transcription factors. The original approach relies on the ectopic expression of the factors Oct4, Sox2, Klf4 and c-myc—collectively known as OSKM^{1–3}. The relative flaws in the understanding of the molecular mechanisms governing induced pluripotent stem (iPS) cells generation hinder the efficient derivation of reprogrammed cells without genetic manipulation. Whereas the modulation of the initial OSKM cocktail with other factors has been extensively documented, comparatively little is known about soluble molecules promoting the process, even if such recombinant factors could be highly valuable for therapeutic applications. Several processes acting as reprogramming roadblocks harbour tumour-suppressive activity such as programmed cell death (PCD) and senescence^{4–6}. As an example, the p53/Puma axis limits iPS cell generation at the late stage of reprogramming and has been related to the dual pro-oncogenic and pro-PCD effect of c-Myc⁷. However, the mechanisms governing cell death independently of p53/c-Myc in the early days of reprogramming remain unclear^{4–6}.

Here we identify the Netrin-1 and its dependence receptor DCC (Deleted in Colorectal Carcinoma) as regulators of somatic cell reprogramming to pluripotency. Netrin-1 is a secreted laminin-related molecule initially identified as an axon guidance cue and more recently proposed as a multifunctional protein implicated both during nervous system development and adult pathologies^{8,9}. Of interest in the scope of this study, Netrin-1 was shown to act as an oncogene by limiting apoptosis induced by its main dependence receptors DCC and UNC5s (UNC5a, UNC5b, UNC5c and UNC5d)^{8–11}. Using a strategy centred on the genomic regions differentially bound by OSKM in somatic and pluripotent cells, we identify members of the Netrin-1/DCC signalling pathway as putative reprogramming roadblocks. We therefore examine Netrin-1 function during iPS cell generation and reveal that the early stage of the process is associated with a transient Netrin-1 transcriptional repression mediated by Oct4 and Klf4 repressive action on the Netrin-1 promoter. We show that such Netrin-1 deficiency limits reprogramming by engaging DCC-induced apoptosis. In parallel, we demonstrate that Netrin-1 protects established pluripotent cells from apoptosis induced by its receptor UNC5b. Importantly, we demonstrate that the Netrin-1/DCC imbalance is corrected by the non-invasive treatment with recombinant Netrin-1 that improves reprogramming efficiency of human and mouse somatic cells.

Results

Netrin-1 level controls iPS cell generation. To identify novel pluripotent reprogramming impediments, we developed a method centred on the OSKM ‘differentially bound regions’ (DBRs; Fig. 1a)¹². DBR were identified by comparing the chromatin immunoprecipitation (ChIP)-Sequencing data for OSKM binding in fibroblasts 48 h after OSKM induction and in established pluripotent cells. This approach led to the identification of 264 ‘DBR genomic regions’ harbouring a differential OSKM binding in both cell types¹². We selected the 705 genes located within DBR as candidate genes potentially hindering pluripotent reprogramming (Fig. 1a). We hypothesized that the partial OSKM binding will delay or abrogate their regulation upon reprogramming and might therefore alter iPS cell generation. By performing gene ontology analysis centred on ‘PCD’, a major reprogramming impediment, we restricted the list to 51 candidates (Step1). Next, 29 candidates were selected

(Step2) due to their dynamic expression during mouse embryonic fibroblasts (MEFs) reprogramming (Supplementary Table 1)¹³. The candidates were subjected to a protein interaction network prediction that revealed an unexpected over-representation of members of the Netrin-1 (Ntn1) signalling pathway with genes corresponding to the Netrin-1 ligand and its receptors DCC, UNC5c and UNC5d (Fig. 1b and Supplementary Table 1).

Ntn1 expression was assessed during MEF reprogramming induced by OSKM retroviral infection and in the resulting iPS cells grown in 2i/LIF (leukemia inhibitory factor) culture conditions. Such approach revealed a biphasic *Ntn1* expression profile with a marked downregulation during the first 6 days of pluripotent reprogramming followed by a strong reactivation in iPS cells (Fig. 1c). The same pattern, even though with a faster kinetic, is observed in OSKM dox-inducible reprogrammable MEF, ruling out possible side effects of retroviral infection on gene expression (Supplementary Fig. 1a)¹⁴.

The dynamic expression of Netrin-1 during reprogramming prompted us to investigate whether this factor regulates iPS cell generation. Oct4/GFP knock-in MEF were infected with lentiviral particles encoding a scrambled short hairpin RNA (shRNA) or three different shRNA-targeting Netrin-1 2 days before reprogramming induction by OSKM retroviral infection and 2i/LIF culture conditions (Fig. 1d and Supplementary Fig. 1b). Netrin-1 depletion led to a significant decrease in iPS cell generation efficiency, as denoted by counting GFP-positive colonies activating endogenous Oct4 promoter (Fig. 1e,f). Similar results were obtained by alkaline phosphatase (AP)-positive colony counting, further confirming Netrin-1 effect on iPS cells generation (Supplementary Fig. 1c,d).

Recombinant Netrin-1 improves reprogramming efficiency.

Conversely, Netrin-1 downregulation at the early stage of reprogramming prompted us to ask whether the non-invasive treatment with soluble recombinant Netrin-1 (rNetrin-1) could compensate for such endogenous deficiency and improve reprogramming features. We induced Oct4/GFP MEF to reprogramme using non-integrative RNA Sendai virus and 2i/LIF culture conditions. In such settings, the treatment with rNetrin-1 led to a eightfold increase in the number of Oct4/GFP-positive colonies (Fig. 1g,h). A similar trend was observed with other reprogramming strategies such as OSKM retroviral infection (Supplementary Fig. 1e–g). Conversely, and as expected, a Netrin-1-blocking antibody reduces AP + colony formation (Supplementary Fig. 1h,i).

We then asked whether Netrin-1 effect on iPS cell generation was restricted to MEFs or could be broadened to other adult somatic cells. We derived various primary cells and detected comparable Netrin-1 expression levels in adult fibroblasts from ear (AEF) or tail tip (ATF) and in intestinal epithelium where Netrin-1 is involved in cell survival and homeostasis (Fig. 1i)¹⁰. Interestingly, OSKM expression induced by doxycycline (dox) treatment in cultured intestinal epithelium led to the same transient *Ntn1* downregulation than in MEF (Fig. 1j), whereas such phenomenon was not observed in AEF (Supplementary Fig. 1j). Next, we plated intestinal epithelium on irradiated feeder and iPS cells formation by dox treatment. As reported, AP-positive iPS colonies reactivating the naive pluripotency marker Nanog emerged around day 15 (Fig. 1k and Supplementary Fig. 1k)¹⁴. In such context, we demonstrated that Netrin-1 knockdown, before reprogramming induction, reduced by 75% the number of Nanog-expressing iPS colonies, whereas recombinant Netrin-1 (rNetrin-1) treatment increased iPS cells generation by a fourfold factor (Fig. 1l,m). Netrin-1 depletion also reduced iPS cell generation efficiency from AEF (Fig. 1n).

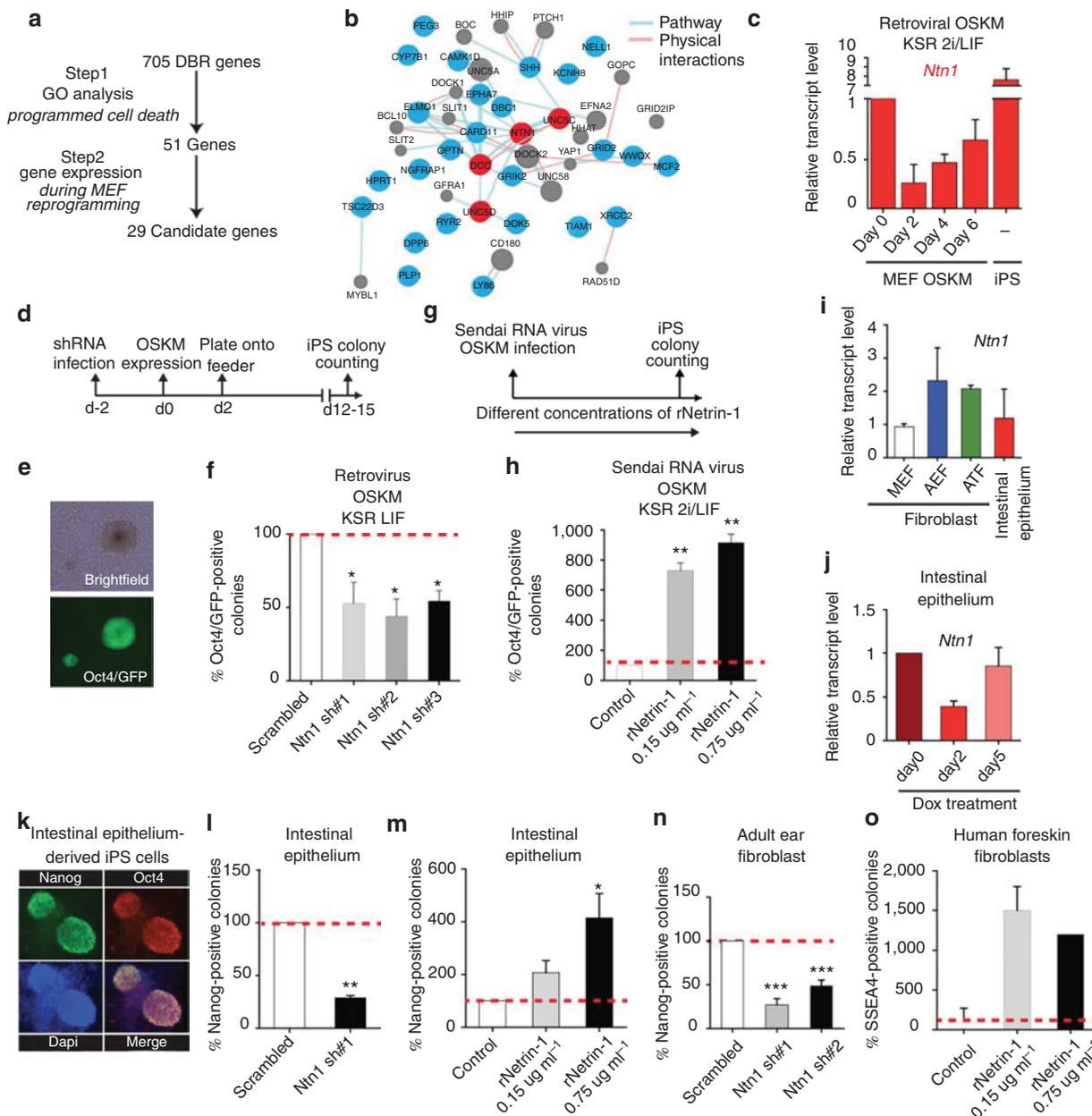


Figure 1 | Netrin-1 level governs reprogramming. (a) Identification of pluripotent reprogramming impediments. DBR genes were selected using UCSC genome browser (<http://genome.ucsc.edu>), gene ontology performed with DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>; gene ontology:0008624, 0006917, 0012502, 0043065). Candidate list was refined to 29 genes using RNA-Seq, microarray data and literature. (b) Predicted protein interaction network (<http://www.genemania.org>). Blue and red dots correspond to candidates, grey dots to potential partners. (c) *Ntn1* expression is biphasic during reprogramming. Quantitative reverse transcription-PCR (Q-RT-PCR) depicts *Ntn1* expression levels during reprogramming induced by OSKM retroviral expression. Data are expressed relative to MEF as the mean \pm s.d. ($n = 3$). (d) Schematic of the time schedule of reprogramming experiment. (e) Typical mouse Oct4/GFP iPS colony 14 days following OSKM infection. Scale bars, 80 μ m. (f) Netrin-1 depletion reduces reprogramming efficiency. The number of Oct4/GFP-positive colonies produced from sh-scrambled MEFs is set at 100% for each individual experiment. Data are the mean \pm s.d. ($n = 3$). Student's *t*-test, $P < 0.05$. (g) Schematic of the time schedule of reprogramming experiment. (h) Recombinant Netrin-1 (rNetrin-1) effect on mouse Oct4/GFP iPS cells generation induced by OSKM RNA sendai virus. rNetrin-1 (0.15 or 0.75 μ g ml⁻¹) was added daily to the media. Data are the mean \pm s.d. ($n = 2$). Student's *t*-test, $P < 0.01$. (i) Netrin-1 expression in various somatic cells. Q-RT-PCR depicts *Ntn1* level in mouse embryonic fibroblast (MEF), adult ear (AEF) and tail tip (ATF) fibroblasts and intestinal epithelium. Data are expressed relative to MEF as the mean \pm s.d. ($n = 3$). (j) Netrin-1 expression is biphasic during intestinal epithelium reprogramming. Q-RT-PCR depicts *Ntn1* expression at day 2 and day 5 post OSKM induction (dox treatment). Data are expressed relative to untreated intestinal epithelium at day 2 and day 5 as the mean \pm s.d. ($n = 2$). (k) Oct4/Nanog immunostaining of a miPS colony derived from intestinal epithelium. Scale bars, 100 μ m. (l) Netrin-1 depletion reduces reprogramming efficiency of intestinal epithelium. The number of Nanog-positive colonies produced from sh-scrambled sample is set at 100%. Data are the mean \pm s.d. ($n = 2$). Student's *t*-test, $**P < 0.01$. (m) Recombinant Netrin-1 (rNetrin-1) effect on iPS cells generation from mouse intestinal epithelium. Data are the mean \pm s.d. ($n = 2$). Student's *t*-test, $*P < 0.05$. (n) Netrin-1 depletion reduces reprogramming efficiency of adult ear fibroblasts (AEFs). Nanog immunostaining was performed after 12–14 days of reprogramming. Data are the mean \pm s.d. ($n = 2$). Student's *t*-test, $***P < 0.001$. (o) Recombinant Netrin-1 effect on human iPS colonies emergence from human foreskin fibroblasts. SSEA4-positive hiPS colonies were counted day 26 post infection.

We next assessed whether recombinant Netrin-1 treatment improves human somatic cells reprogramming. Treatment of human foreskin fibroblasts (HFFs) with rNetrin-1 induced to reprogramme by non-integrative RNA Sendai virus led to a 15-fold increase in reprogramming efficiency, as evaluated by counting the number of SSEA4-positive colonies (Fig. 1o). These findings identify (i) Netrin-1 as a novel factor governing iPS cell formation and (ii) recombinant Netrin-1 as a novel soluble factor improving reprogramming efficiency of various human and mouse somatic cells.

In a therapeutic perspective of improving reprogramming efficiency, we addressed whether recombinant Netrin-1 (rNetrin-1) treatment is detrimental to the iPS cell quality. We characterized pluripotency features and genomic stability of mouse (miPS) and human iPS (hiPS) cells derived with rNetrin-1. We expanded mouse Oct4/GFP iPS (miPS) clones obtained with integrative and non-integrative methods in standard conditions ('control') or with rNetrin-1 ($0.6 \mu\text{g ml}^{-1}$; 'rNetrin-1 derived'). The 9 'rNetrin-1 derived' miPS clones generated using RNA sendai virus harboured similar levels for *Nanog*, *Rex1* and *Esrrb* RNA than the three 'control' miPS clones and mouse embryonic stem (ES) cells, showing that rNetrin-1 is not detrimental to the transcriptional reactivation of such naive pluripotent circuitry (Fig. 2a). In parallel, we performed RNA-sequencing analysis to compare Oct4/GFP MEF, partially reprogrammed (pre-) miPS cells, 'control' miPS cells and two different 'rNetrin-1 derived' miPS clones generated by OSKM retroviral infection (Fig. 2b and Supplementary Fig. 2a). The mouse pre-iPS cells do not reactivate the Oct4/GFP endogenous locus and do not silence the retroviral transgenes¹⁵. The calculation of the euclidian distances revealed a strong correlation between the 'control' and the two 'rNetrin-1-derived' miPS clones, confirming similar transcriptome resetting in both conditions (Fig. 2b). In particular, 'rNetrin-1-derived' miPS cells harbour similar *Nanog*, *Esrrb* and *Dppa3* levels than 'control' miPS cells, whereas the pre-iPS cells failed at reactivating these genes (Supplementary Fig. 2b). After 40 passages in culture, the quantification of major chromosomal alterations did not reveal any significant difference between miPS cell lines, suggesting that rNetrin-1 treatment does not impact massively chromosomal stability (Supplementary Fig. 2c). In terms of differentiation potential, we showed that 'rNetrin-1-derived' miPS cells turn off the endogenous Oct4/GFP promoter activity following LIF withdrawal for 5 days (Supplementary Fig. 2d). Embryoid body formation assays demonstrated their ability to give rise to derivatives of the three germ layers (Fig. 2c). The histological analysis of teratoma formed by 'rNetrin-1 derived' miPS cells confirmed the presence of tissues representing all three germ layers (Fig. 2d). In parallel, two hiPS cell lines derived from foreskin fibroblasts with rNetrin-1 were characterized at the molecular and functional levels. As shown in Fig. 2e,f, 'rNetrin-1 derived' hiPS cells express the pluripotency-associated markers OCT4, TRA1-60 and SSEA4. Karyotype analysis revealed no abnormalities and teratoma formation confirmed the presence of derivatives of the three germ layers (Fig. 2g,h). Altogether, these results suggest that the treatment with recombinant Netrin-1 is not detrimental to mouse and hiPS cell features.

Netrin-1 limits DCC-induced apoptosis during reprogramming. To decipher the mechanisms downstream of Netrin-1, the expression of its receptors was assessed during MEF reprogramming. In the first 6 days, DCC expression remains steady, whereas *UNC5b* and *UNC5c* are rapidly downregulated (Fig. 3a and Supplementary Fig. 2e). DCC belongs to the functional family of dependence receptors sharing the ability of triggering apoptosis in the absence of their ligand (Fig. 3b). The expression

of such receptor thus renders the cell dependent for its survival on ligand availability^{8,16,17}. In Netrin-1-limiting conditions, unbound DCC triggers apoptosis via a cleavage of its intracellular domain¹⁸. We asked whether Netrin-1 transient downregulation limits reprogramming by engaging DCC-induced apoptosis. To test this hypothesis, DCC expression was silenced with shRNA lentiviral particles before MEF reprogramming induced by OSKM retroviral infection. DCC knockdown using three different shRNA significantly improves reprogramming efficiency, as denoted by counting the number of Oct4/GFP colonies (Fig. 3c,d and Supplementary Fig. 2f). More importantly, the Netrin-1 depletion effect is rescued by DCC knockdown, identifying the pair Netrin-1/DCC as a novel mediator of reprogramming (Fig. 3e). In contrast, silencing of the Netrin-1 receptor *UNC5b* did not modify iPS cell generation efficiency (Supplementary Fig. 2g,h). We generated a mice model in which a DCC point mutation at the critical amino acid required for intracellular cleavage (aspartic acid 1290) is sufficient to block DCC-induced apoptosis while leaving unaffected the positive DCC signalling observed upon Netrin-1 binding (Fig. 3f)¹⁹. MEFs derived from *DCC^{wt/wt}* and *DCC^{D1290N/D1290N}* littermate embryos were reprogrammed by retroviral OSKM infection and 2i/LIF culture conditions. *DCC^{D1290N/D1290N}* MEFs gave rise to iPS cell colonies with a slightly higher efficiency than *DCC^{wt/wt}* MEFs, indicating that DCC effect on reprogramming efficiency is at least partly due to its pro-apoptotic activity (Fig. 3g). Recombinant Netrin-1 (rNetrin-1) effect was compared during *DCC^{wt/wt}* and *DCC^{D1290N/D1290N}* MEF reprogramming. The rNetrin-1 and Netrin-1 blocking antibody effects are nearly abrogated in *DCC^{D1290N/D1290N}* MEF, confirming that Netrin-1 effect is partly mediated by the inhibition of DCC pro-apoptotic activity (Supplementary Fig. 2i,j).

Reprogramming factors, especially c-Myc, activate the p53 pathway whose alleviation reduces apoptosis and improves iPS cells generation⁴⁻⁶. We investigated whether Netrin-1 effect was dependent on c-Myc/p53. First, reprogramming experiments were performed without c-Myc (OSK) using integrative and non-integrative methods⁴. We demonstrated that, in the absence of c-Myc, the modulation of Netrin-1 level still impacts iPS cell generation, even if at a lesser extent (Supplementary Fig. 2k,l). To assess whether Netrin-1 effect was p53 mediated, *p53^{-/-}* MEFs were infected with shRNA against *Ntn1* before OSKM reprogramming. Such approach still led to a significant decrease in the number of AP-positive colonies (Fig. 3h,i). The treatments with rNetrin-1 or Netrin-1 blocking antibody are also still effective in the absence of p53 (Supplementary Fig. 2m,n). Moreover, p53 protein levels remained unchanged by rNetrin-1 treatment at reprogramming day 4 (Fig. 3j). Taken together, these data support the view that DCC pro-apoptotic activity limits pluripotent reprogramming mainly in a c-Myc/p53-independent manner.

We next analysed apoptosis occurrence at the early stage of reprogramming by FACS combining the cell surface markers *thyl* and SSEA1 with 4,6-diamidino-2-phenylindole (DAPI) and Annexin V^{15,20,21}. The starting MEF population express *thyl*, whereas SSEA1 is repressed (*thyl^{Positive}/SSEA1^{Negative}*). FACS analysis performed at days 3 and 5 post OSKM infection confirmed the emergence of a *thyl^{Negative}/SSEA1^{Negative}* subpopulation (*thyl^{Neg}*) enriched in reprogramming cells, as shown by morphology and AP staining (Fig. 4a and Supplementary Fig. 3a). We showed that, in the early days of reprogramming, Netrin-1/DCC ratio modulation directly impacts apoptosis occurrence with *thyl^{Neg}* cells being particularly sensitive to such phenomenon (Fig. 4b,c and Supplementary Fig. 3b-e). Following *Ntn1* silencing, the percentage of *thyl^{Neg}* apoptotic cells is increased by 30% at reprogramming day 3 (Fig. 4b,c and

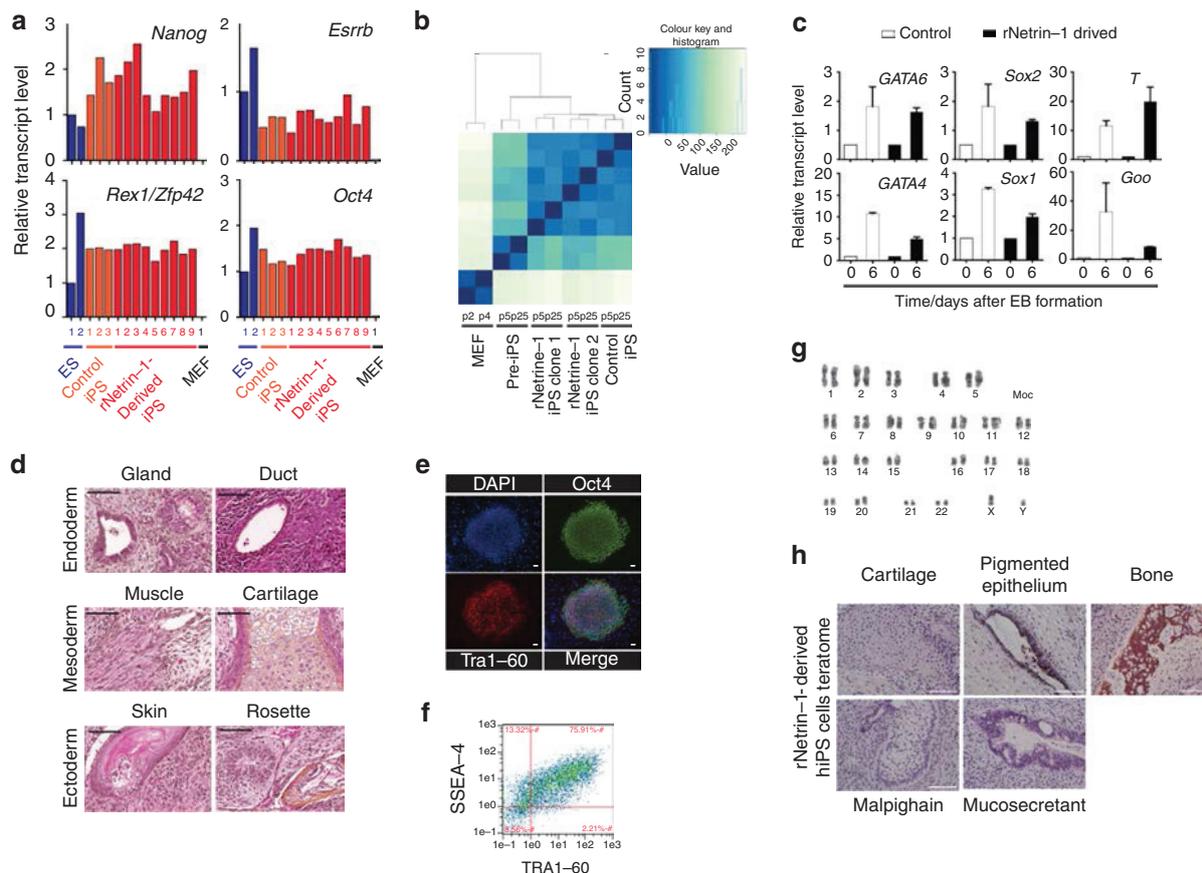


Figure 2 | Recombinant Netrin-1 treatment is not detrimental to iPS cells quality. (a) Naive pluripotency markers expression in ‘control’ and ‘rNetrin-1 derived’ miPS cells from RNA Sendai virus. Mouse ES cells, three ‘control’ iPS clones, nine ‘rNetrin-1-derived’ iPS clones and MEF were analysed for *Nanog*, *Esrrb*, *Rex1/Zfp42* and *Oct4* expression by quantitative reverse transcription-PCR. (b) Heatmap of the euclidean distances between the samples as calculated from the variance stabilizing transformation of the count data (DESeq). Samples consist of Oct4/GFP MEF, mouse pre-iPS, ‘control’ and ‘rNetrin-1-derived’ iPS clones derived by OSKM retroviral infection. (c) ‘Control’ and ‘rNetrin-1-derived’ mouse iPS cells form embryoid bodies containing derivatives of the three germ layers. Data are normalized to housekeeping genes and expressed relative to t0 as the mean \pm s.d. ($n=3$). (d) Teratoma histological analysis from ‘rNetrin-1-derived’ miPS cells showing contribution to the three germ layers. Scale bars, 100 μ m. (e) Pluripotency markers expression in ‘rNetrin-1-derived’ human iPS cells. Immunostainings for OCT4 and Tra1-60 were performed on a hiPS colony. Scale bars, 50 μ m. (f) SSEA4 and TRA1-60 FACS analysis of ‘rNetrin-1-derived’ hiPS cells. (g) Karyotype of ‘rNetrin-1-derived’ hiPS cells. (h) Histological analysis of a ‘rNetrin-1-derived’ hiPS cells teratoma. Scale bars, 200 μ m.

Supplementary Fig. 3e). Conversely, following DCC knockdown, the percentage of $thy1^{Neg}$ apoptotic cells is decreased by 20% and 40% at reprogramming day3 and 5, respectively. As expected, *Ntn1* and DCC double knockdown led to a reduction in apoptosis occurrence (Fig. 4b,c and Supplementary Fig. 3e). Taken together, these data support the view that the early Netrin-1/DCC imbalance limits iPS generation by inducing DCC-mediated apoptosis predominantly in $thy1^{Neg}$ reprogramming cells. *Ntn1* and *DCC* expression levels were next assessed at this stage of reprogramming. At day 3, *Ntn1* expression is strongly downregulated in $thy1^{Neg}$ cells, whereas *DCC* remains steady—situation favouring DCC-mediated cell death (Fig. 4d). However, 3 days later, the pattern is inverted with *Ntn1* reactivation and *DCC* silencing, possibly reflecting the elimination of the DCC-expressing cells (Fig. 4d). The *Ntn1* activation is maintained at day 9 in the $thy1^{Negative}/SSEA1^{Positive}$ cells co-expressing *Nanog* and in established iPS cells (Supplementary Fig. 3f)^{15,21}.

Oct4, Klf4 and the NuRD complex repress Netrin-1 expression. We then asked which epigenetic mechanism triggers Netrin-1 transient transcriptional repression. In agreement with its

location within DBR, the *Ntn1* locus is occupied by Oct4, Sox2 and Klf4 in mouse ES cells (mm8 genome browser: Oct4-binding chr11: 68210308-68210332, Sox2-binding chr11: 68210051-68210500, Klf4-binding chr11: 68210297-68210303; Supplementary Fig. 4a)^{12,22}. We evaluated OSKM effect on the activity of Netrin-1 promoter²³. Luciferase assays performed on OSKM dox-inducible MEF transfected with different promoter constructs demonstrated the ability of the reprogramming cocktail to repress specifically Netrin-1 promoter activity, whereas control SV40 promoter activity remains steady (Fig. 4e,f). A similar approach was employed in HEK293T cells with single reprogramming factors to reveal that Oct4 and Klf4 are responsible for Netrin-1 promoter repression (Fig. 4g).

Different epigenetic complexes, such as Polycomb, WDR5 and NuRD, are associated with early development and connected to the reprogramming factors activity^{24–27}. Among them, the nucleosome remodelling and deacetylase (NuRD) complex member Mbd3 has been identified as a strong reprogramming roadblock, with OSKM inducing a global redistribution of Mbd3 on target genes in MEF (1,177 binding regions in MEF compared with 8,657 after OSKM induction)²⁶. ChIP-Sequencing data revealed that, upon dox induction in MEF, Mbd3 gets specifically

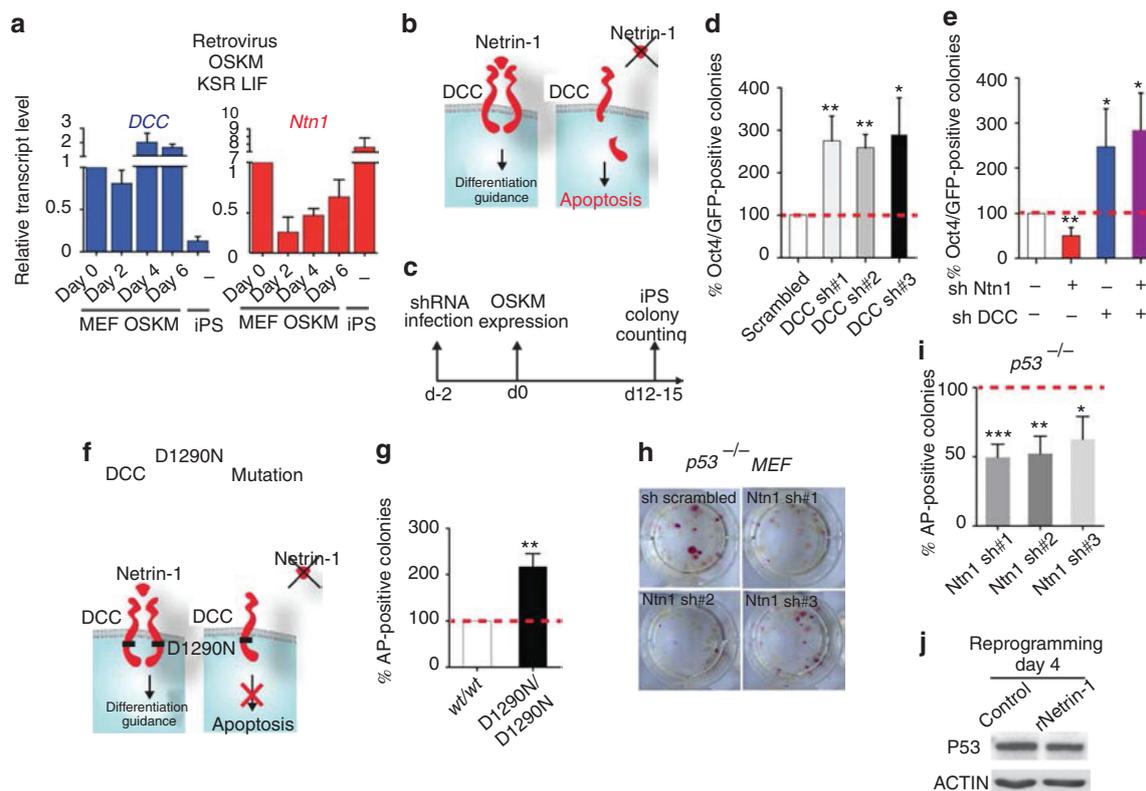


Figure 3 | DCC pro-apoptotic activity limits reprogramming occurrence. (a) DCC expression is maintained at the early stage of reprogramming. Quantitative reverse transcription-PCR depicts *DCC* and *Ntn1* expression profile during reprogramming. Data are expressed relative to MEF as the mean \pm s.d. ($n=3$). (b) Schematic depicting the dependence receptor paradigm. In presence of its ligand Netrin-1, DCC transduces a positive signalling leading to differentiation and guidance cues. In the absence of or in Netrin-1-limiting conditions, DCC actively induces caspase-dependent apoptosis. (c) Schematic of the time schedule of reprogramming experiment. (d) DCC depletion improves reprogramming efficiency. The number of Oct4/GFP colonies produced from sh-scrambled MEFs is set at 100% for each individual experiment. Data are the mean \pm s.d. ($n=3$). Student's *t*-test, $*P<0.05$ or $**P<0.01$. (e) Netrin-1 depletion effect is rescued by DCC knockdown. Data are the mean \pm s.d. ($n=3$). Student's *t*-test, $*P<0.05$ or $**P<0.01$. (f) Scheme depicting *DCC*^{D1290N} point mutant model. In the presence of Netrin-1, DCC-positive signalling remains unaffected. In settings of ligand absence, DCC pro-apoptotic activity is abrogated. (g) DCC pro-apoptotic activity abrogation improves reprogramming efficiency. MEFs were derived from embryos of *DCC*^{wt/D1290N} mice intercrosses. *DCC*^{wt/wt} and *DCC*^{D1290N/D1290N} were subjected to reprogramming and AP-positive colonies scored at days 12–14. The number of colonies from *MEF*^{wt/wt} is set at 100% for each individual experiment. Data are the mean \pm s.d. ($n=3$). Comparable transduction efficiencies were achieved with *DCC*^{wt/wt} and *DCC*^{D1290N/D1290N} MEF. Student's *t*-test, $*P<0.05$. (h–j) Netrin-1 effect is mainly p53 independent. (h–i) Netrin-1 depletion reduces reprogramming efficiency of *p53*^{-/-} MEF. Data are the mean \pm s.d. ($n=3$). Student's *t*-test, $*P<0.05$ or $**P<0.01$ or $***P<0.001$. (j) p53 protein levels are not modulated by rNetrin-1 treatment. Western blot for p53 and ACTIN was performed on MEF treated with dox for 4 days in the presence or absence of recombinant Netrin-1 (0.75 $\mu\text{g ml}^{-1}$).

recruited on the Netrin-1 promoter, at a genomic location that corresponds exactly to Oct4-, Sox2- and Klf4-binding site in mouse ES cells (mm8 genome browser: Mbd3-binding chr11: 68209776–68210746; Fig. 4h)¹². As depicted in Fig. 4i, Mbd3 knockdown before reprogramming induction led to a rescue of *Ntn1* downregulation. In agreement with this finding, comparison of Netrin-1 expression profile during *MEF*^{wt/wt} and *Mbd3*^{fl/fl} reprogramming shed light on a significantly higher induction of *Ntn1* in day 8 *Mbd3*^{fl/fl} MEF reprogramming than in day 11 *MEF*^{wt/wt}, whereas *Ntn3* expression profile remains unaffected (Supplementary Fig. 4b). Luciferase assays confirmed that Oct4 and Klf4 repressive effect on Netrin-1 promoter activity is mainly mediated by Mbd3 (Fig. 4j). Moreover, depletion of the other NuRD components Mta1 and Chd4 before reprogramming also rescued *Ntn1* downregulation, whereas Mta2 knockdown had no effect (Fig. 4k). Taken together, these data demonstrate that a Mbd3/Mta1/Chd4-containing NuRD complex is responsible for the transient Netrin-1 transcriptional repression observed upon reprogramming.

Netrin-1 limits apoptosis in pluripotent cells. Finally, owing to the high Netrin-1 expression level in iPS cells, we asked whether Netrin-1 contributes to pluripotency maintenance. Taking advantage of blastocyst-derived stem cells *in vitro*, we showed that *Ntn1* expression is confined to ES cells and absent from the extra-embryonic-derived TS and XEN cells, whereas *UNC5b* is detected in the three *in vitro* cellular models, even if at a reduced level compared with MEF (Fig. 5a). We next showed that the Netrin-1 promoter activity is strong in pluripotent ES cells compared with MEF, because of genomic elements located in the promoter part B (Fig. 5b). We asked whether Netrin-1 is expressed during pre-implantation development but *Ntn1* mRNA could not be detected in blastocysts by *in situ* hybridization. This observation was confirmed by exploiting single-cell PCR resource performed in mouse blastomeres and in ES cells²⁸. *Ntn1* and its receptors (*DCC*, *UNC5a-d*) are not detected at the blastocyst or epiblast stages *in vivo* but *Ntn1* and *UNC5b* are activated during the ES cell derivation process (Supplementary Fig. 4c). To evaluate Netrin-1 function, Netrin-1 knockdown ES cells were

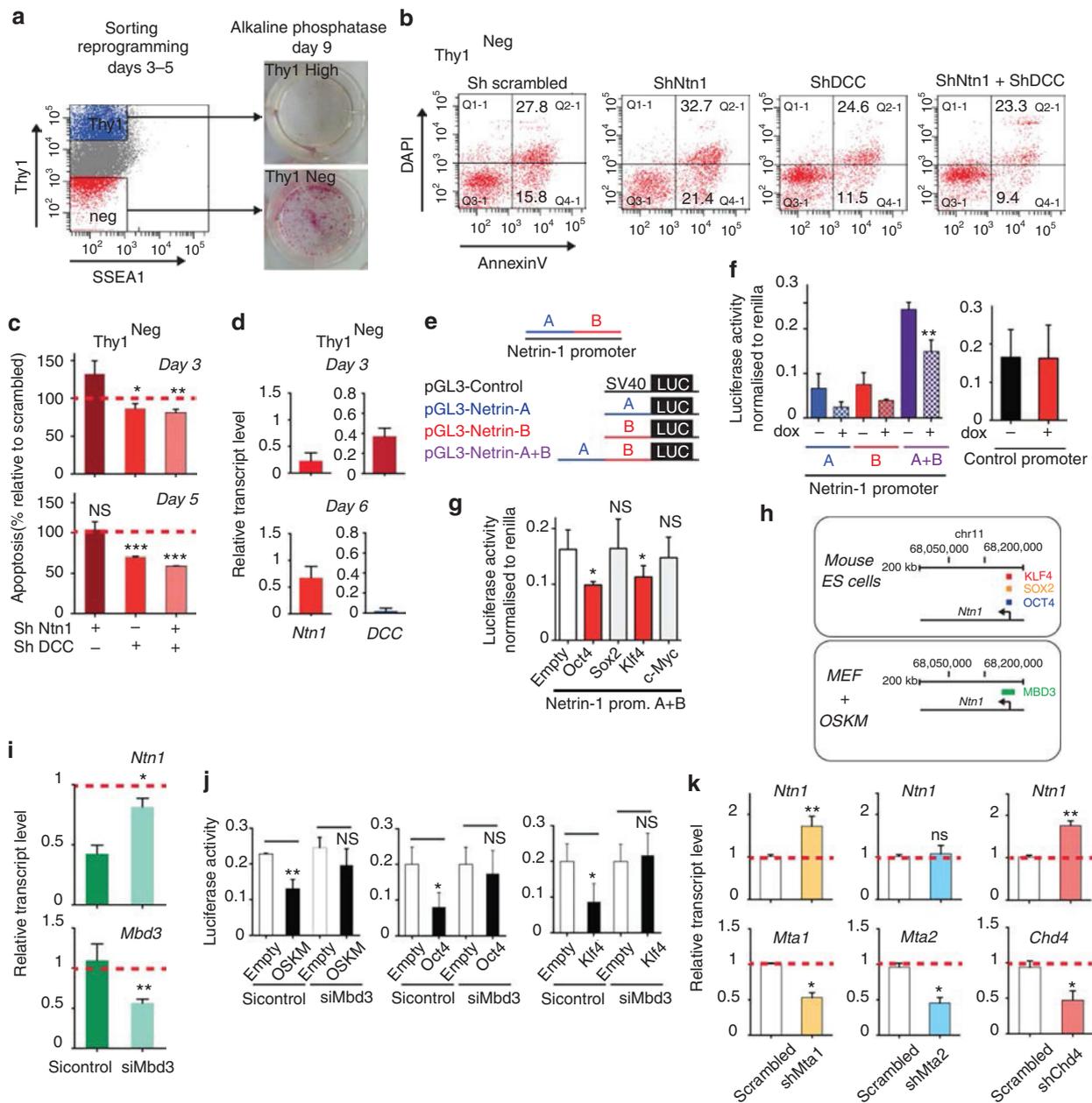
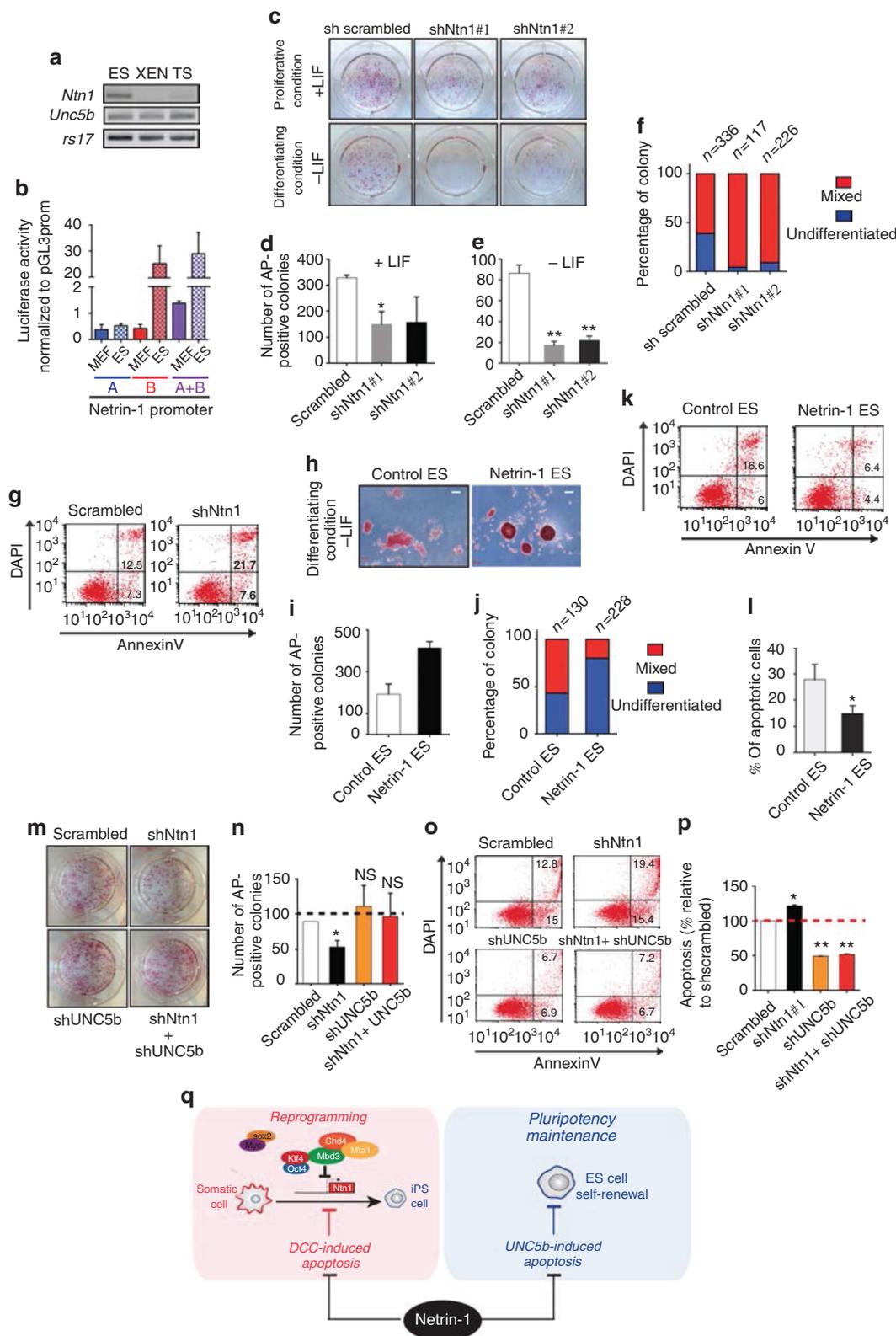


Figure 4 | Ntn1/DCC balance modulates apoptosis at the early stage of reprogramming. (a) Representative FACS profile of thy1^{High} and thy1^{Neg} cells 3–5 days post OSKM infection. Cells were sorted and plated back in reprogramming media for 6–9 additional days before AP activity staining. (b) Representative FACS profile of apoptosis in thy1^{Neg} cells following Ntn1 and/or DCC depletion. Apoptotic cells percentages are indicated. (c) Quantification of thy1^{Neg} apoptotic cells following Ntn1 and/or DCC depletion at days 3 and 5. The apoptotic cell percentage in sh-scrambled thy1^{Neg} MEFs is set at 100%. Data are the mean ± s.d. (n = 3). Student’s *t*-test, **P* < 0.05 or ***P* < 0.01 or ****P* < 0.001; NS, non-significant. (d) *Ntn1* and *DCC* expression levels in thy1^{Neg} cells. Cells were sorted at days 3 and 6 post OSKM retroviral infection. Quantitative reverse transcription-PCR (Q-RT-PCR) data are expressed relative to MEF as the mean ± s.d. (n = 3). (e) Scheme depicting Netrin-1 promoter constructs: part A, B and A + B²³. pGL3-control vector with SV40 promoter and enhancer driving luciferase expression is used as ‘control vector’. (f) Dox-inducible MEFs were transfected with Netrin-1 promoter luciferase constructs and treated with or without dox for 48 h. Data are normalized to Renilla activity and expressed as the mean ± s.d. (n = 2). Student’s *t*-test, *P* < 0.05. (g) Regulation of Netrin-1 promoter activity by single reprogramming factors. HEK293T cells were transfected with Netrin-1 promoter part A + B and reprogramming factors. Data are the mean ± s.d. (n = 3). Student’s *t*-test, *P* < 0.05. (h) Mbd3 is recruited to Ntn1 promoter upon reprogramming. ChIP-seq resource was employed to assess Ntn1 promoter occupancy in mouse ES cells and in MEF following OSKM induction. Data are extracted from refs ^{22,26}. Mbd3 is recruited on chromosome 11 (position 68209776–68210746 NCBI36/mm8) in the vicinity of Oct4- (chr11 68210308–68210332), Sox2- (chr11 68210051–68210500) and Klf4- (chr11 68210297–68210303) binding sites. (i) Netrin-1 transcriptional silencing is rescued by Mbd3 depletion. MEFs were transfected with Mbd3 siRNA 24 h before OSKM infection and cells harvested 36 h post OSKM infection. Data are the mean ± s.d. (n = 3). Student’s *t*-test, **P* < 0.05 or ***P* < 0.01. (j) OSKM repressive effect on Ntn1 promoter activity is mediated by Mbd3. HEK293T cells were transfected with Mbd3 siRNA before Ntn1 promoter luciferase construct and reprogramming factors. Luciferase activity was assessed 48 h post transfection and normalized to Renilla activity. Data are the mean ± s.d. (n = 2). Student’s *t*-test, **P* < 0.05. (k) Netrin-1 silencing is mediated by Mta1 and Chd4. MEFs were infected with lentiviral particles targeting the NuRD complex members Mta1, Mta2 and Chd4 and reprogramming induced 48 h later. Q-RT-PCR depicts *Ntn1*, *Mta1*, *Mta2* and *Chd4* expression levels at reprogramming day 2. Data are the mean ± s.d. (n = 3). Student’s *t*-test, **P* < 0.05 or ***P* < 0.01.

established by lentiviral infection with two different shRNA (Fig. 5c–g and Supplementary Fig. 4d). In a colony formation assay, we showed that Netrin-1 depletion led to a drastic reduction in the number of AP-positive colonies obtained in the presence or absence of LIF, demonstrating that Netrin-1 contributes to maintain ES cell self-renewal properties (Fig. 5c–e).

Moreover, when the morphology of the mouse ES colonies was compared, a 30% decrease in the percentage of ‘undifferentiated’ colonies (round, strongly and homogeneously positive for AP) and a concomitant 30% increase in the percentage of ‘mixed’ colonies (weak and heterogeneous AP staining) was detected with the Netrin-1-depleted population, suggesting a defect in the



stability of the pluripotent state (Fig. 5f). This phenotype could be due to a defect in cell cycle and/or survival in such challenging conditions. No significant change on cell cycle could be detected upon Netrin-1 knockdown (Supplementary Fig. 4e). However, Netrin-1 depletion led to a significant increase of the percentage of apoptotic cells, whereas necrotic occurrence remains unaffected, demonstrating a protective effect of Netrin-1 in pluripotent cells (Fig. 5g and Supplementary Fig. 4f). Conversely, stable mouse ES clones expressing exogenously Netrin-1 present an improved self-renewal ability in colony formation assay (Fig. 5h–j). The effect on the ES cell morphology was particularly striking with colonies that remained undifferentiated even after 5 days in differentiation conditions in the absence of LIF (Fig. 5h). Interestingly, such Netrin-1 exogenous expression reduced significantly apoptosis occurrence in ES cells (Fig. 5k,l and Supplementary Fig. 4g). Owing to *UNC5b* expression in ES cells, we asked whether Netrin-1 function was mediated by this dependence receptor. *UNC5b* knockdown rescues Netrin-1 depletion effect on colony formation assay and apoptosis occurrence, identifying the pair Netrin-1/*UNC5b* as novel regulators of apoptosis in pluripotent ES cells *in vitro* (Fig. 5m–p and Supplementary Fig. 4h).

Discussion

Together, the data presented here described the importance of the dependence receptor paradigm both in the regulation of reprogramming and of pluripotency, the balance between Netrin-1 and its receptors DCC or *UNC5b* being key to regulate both processes. Dependence receptors form a family of more than 20 membrane receptors that are not linked by their structure, but by the common functional trait of triggering active apoptosis in the absence of their respective ligand. Thus, cells expressing these types of receptor are dependent on the presence of ligands in the extracellular environment to survive.

Along this line, it was recently formerly demonstrated that the Netrin-1/DCC pair plays a major role in cancer with DCC constraining tumour progression^{19,29} and with Netrin-1-promoting tumour progression^{10,30} via their respective pro-apoptotic/survival activity. This dual activity may also be implicated in neuronal navigation and developmental angiogenesis^{11,31}

In this study, we identified unexpected functions for the secreted cue Netrin-1 in regulating iPS cell generation and pluripotency maintenance (Fig. 5q)²⁶. On the one hand, we propose an integrated model deciphering how the early

dichotomous function of OSKM leads to the recruitment of a repressive Mbd3/Mta1/Chd4-containing NuRD complex to the *Ntn1* promoter, leading to Netrin-1 deficiency. This finding reinforces and broadens the view that early OSKM impaired binding to the somatic genome, combined with the recruitment of antagonistic epigenetic complexes, result in aberrant gene expression that is detrimental for iPS cell generation^{12,26}. We show that the destabilization of the balance between Netrin-1 and its receptor DCC leads to apoptosis induction at the early stage of reprogramming and reduces iPS cell generation. Importantly, we established Netrin-1 as a recombinant protein able to improve the generation of miPS and hiPS cells under specific culture conditions. On the other hand, our work shed light on another Netrin-1 function in ES cell self-renewal, notably by constraining *UNC5b*-mediated apoptosis. Our work, by providing novel insights into the early stage of reprogramming and pluripotency maintenance, should shorten the road to develop clinically useful iPS cells.

Methods

Cell culture and RNAi experiments. MEFs were derived from E13.5 embryos from different backgrounds. Mouse pre-iPS, iPS and *Cgr8* ES cells were cultured on gelatin or on irradiated MEF in KSR + LIF or KSR 2i + LIF³² as previously described³³. Mouse XEN and TS cells were grown as previously reported³³. Intestinal epithelium was grown and induced to form iPS cells, as previously reported¹⁴. 293T and plat-E cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. For reprogramming, when stipulated, 2i components were added 5–6 days post OKM induction (infection or dox treatment). PD0325901 and chiron were purchased from Merck Millipore. shRNA experiments were performed using pLKO.1 vectors and short interfering RNA (siRNA) using esiRNA from Sigma (SHCLNG-NM_008744 for *Ntn1*, SHCLNG-NM_007831 for DCC, SHCLNG-NM_029770 for *UNC5b* and EMU022741 for *Mbd3*, SHCLNG-NM_054081 for *Mta1*, SHCLNG-NM_11842 for *Mta2* and SHCLNG-NM_145979 for *Chd4*). TRC clone numbers are available upon request. Luciferase assays were performed using dual luciferase reporter assay system (Promega, E1910). MEF and HEK293 cells were transfected using Lipofectamine 2000 (Life Technologies). Human *Mbd3* stealth siRNA were employed (Life Technologies, hss147580 and hss147581). The different constructs of human Netrin-1 promoter were already described²³. HA-Flagged Netrin-1 cDNA was cloned into the *PacI* and *XhoI* restriction sites of pcagg-ires-puro plasmid giving rise to pcagg-Netrin-1-ires-puro vector (pcagg-ires-puro is a kind gift from Ian Chambers). ES cells were stably transfected using FugeneHD reagent (Promega, E2691).

Antibodies. Primary antibodies used in this study for immunofluorescence and FACS are as follows: anti-Oct4 (Santa Cruz, C10), anti-Nanog (Cosmobio, RCA B000 2P-F), anti-thy1 (Ebiosciences, 53-2.1), anti-SSEA1 (Stem Cell Technologies, 60060PE) and NL493-conjugated anti-SSEA4 antibody (R&D Systems, SC023), anti-Tra1-60 (Stemgent, 09-0009).

Figure 5 | Netrin-1 promotes pluripotency features *in vitro*. (a) *Netrin-1* and *UNC5b* expression in blastocyst-derived stem cells. Semi-quantitative RT-PCR depicts *Ntn1*, *UNC5b* and housekeeping *rs17* expression levels. (b) Netrin-1 promoter activity in pluripotent versus somatic contexts. Netrin-1 promoter constructs were transfected in mouse embryonic stem (mES) and mouse embryonic fibroblasts (MEF) cells. Data are normalized to Renilla activity and expressed as the mean \pm s.d. ($n = 3$). Student's *t*-test, $P < 0.05$. (c–f) Netrin-1 knockdown decreases colony formation ability of mES cells. sh scrambled, shNtn1#1 and shNtn1#2 mES cells were plated at low density and grown for 6 days in the presence (proliferating condition) or absence (differentiation condition) of LIF before AP staining. (c) Colony formation assay results. (d,e) Counting of AP-positive colonies obtained in the presence (d) or in the absence (e) of LIF. Data are expressed as the mean \pm s.d. ($n = 3$). Student's *t*-test, $P < 0.05$. (f) Netrin-1 depletion affects mES cells pluripotency. 'Undifferentiated' and 'mixed' colonies were scored after colony formation assay. The *n* indicates the number of colonies counted. Data are representative of three independent experiments. (g) Representative FACS profile of apoptosis in sh scrambled and shNtn1#1 mES cells. Cells were plated at low density for 3–5 days and stained with Annexin V/DAPI. (h–j) Netrin-1 exogenous expression improves mES cells self-renewal properties. (h) AP pictures of 'control' and 'Netrin-1-expressing' mES cells. Scale bars, 100 μ m. (i) Counting of AP-positive colonies. Same protocol as c. Data are expressed as the mean \pm s.d. ($n = 3$). (j) 'Undifferentiated' and 'mixed' colonies were scored after colony formation assay. Data are representative of three independent experiments. (k) Representative FACS profile of apoptosis in 'control' and 'Netrin-1-expressing' mES cells. (l) Quantification of apoptosis in 'control' and 'Netrin-1-expressing' ES cells. Data are the mean \pm s.d. ($n = 3$). Student's *t*-test, $*P < 0.05$. (m–p) Netrin-1 depletion effect is rescued by *UNC5b* knockdown. (m) Bright-field pictures of colony formation assay results. (n) AP-positive colony counting. Data are expressed as the mean \pm s.d. ($n = 3$). (o,p) Netrin-1 depletion effect on apoptosis is rescued by *UNC5b* knockdown. (o) Representative FACS profile of apoptosis in sh scrambled, shNtn1, shUNC5b and shNtn1 + shUNC5b mES cells. (p) Quantification of apoptosis following Netrin-1 and/or *UNC5b* depletion. The apoptotic cells percentage in sh-scrambled mES cells is set at 100%. Data are the mean \pm s.d. ($n = 2$). Student's *t*-test, $*P < 0.05$ or $**P < 0.01$. (q) Model recapitulating Netrin-1 function in reprogramming and pluripotency maintenance.

Retroviral and lentiviral production. Plat-E cells were used to produce pMXs-based retroviruses containing the cDNA for Oct4, Sox2, Klf4 or cMyc, as previously described³². PlatE-generated mCherry retrovirus was used to monitor MEF infection efficiency in each experiment. 293T cells were used to produce lentiviral pLKO-derived particles.

Mouse iPS cells generation. For iPS induction, MEFs were seeded per well of a six-well plate and 12 h later overnight infections were performed using equal amounts of each indicated retrovirus in the presence of $8 \mu\text{g ml}^{-1}$ polybrene (Sigma, 107689). The day after, wells were rinsed twice and cells were cultivated in medium supplemented or not with 150 or 750 ng ml^{-1} recombinant Netrin-1 (rNetrin-1). Forty-eight hours after infection, MEFs were reseeded onto irradiated feeders in iPS media. Media were replaced every day with medium alone or freshly supplemented with rNetrin-1. Emerging iPS colonies were monitored until days 12–14 when cells were harvested or individual colonies picked for further analysis. Similar protocol was employed with RNA sendai virus (cytotune 2.0, Life Technologies). The dox-inducible mice were purchased from the Jackson Laboratories (stock number 011001). MEFs, adult fibroblasts from ear and tail tip and intestinal epithelium were plated on irradiated feeders and treated with doxycycline at $2 \mu\text{g ml}^{-1}$. Recombinant Netrin-1 was purchased from Adipogen (AB40B-0075). Because of the poor stability of the recombinant Netrin-1 molecule and variability between batches, batch activity was systematically tested in cell death assays using UNC5b-transfected cells. The blocking Netrin-1 antibody was obtained from the Netris Pharma company.

Human iPS cells generation. Reprogramming experiments have been performed on 5×10^5 HFFs (Millipore) seeded in a well of six-well plates and grown in FibroGRO-LS medium (Millipore) until replating on MEFs. Cells were infected overnight with Sendai viruses (Cytotune, Life Technologies) each of them at multiplicity of infection (MOI) = 3. The day after, wells were rinsed twice and cells were cultivated in medium supplemented or not with 150 or 750 ng ml^{-1} rNetrin-1. Media were replaced every day with medium alone or freshly supplemented with rNetrin-1. Seven days post infection, cells were seeded onto freshly mitomycin-C-treated MEF at a density of 1×10^5 or 2×10^5 HFF cells per plate. The next day medium was switched to DMEM/F12 culture medium supplemented with 20% PluriQ Serum Replacement (GlobalStem), 0.1 mmol l^{-1} non-essential amino acids, 1 mmol l^{-1} L-glutamine, 0.1 mmol l^{-1} 2-mercaptoethanol, penicillin/streptomycin (all of them from Life Technologies), 12.5 ng ml^{-1} recombinant human basic fibroblast growth factor (Milytenyi Biotec). At day 26, fully reprogrammed colonies were manually picked and transferred to freshly mitomycin-C-treated MEF for amplification.

Apoptosis detection. Cells were stained with anti-thy1 and apoptosis quantified with the Dead Cell Apoptosis Kit with Annexin V FITC and DAPI (Life technologies, V13242).

Colony formation assay was performed as previously described³².

FACS sorting. Analysis was performed on a BD LSRFortessa. Sorting was performed on a BD FACSDiVa. Cells were sorted, washed immediately and centrifuged before being plated directly in fresh medium or frozen for RNA extraction and gene expression analysis.

RNA-Sequencing. RNA quality was analysed using a Bioanalyser (Agilent). Libraries were constructed and sequenced on an illumina Hiseq 2000 by the MGX company. GEO record series GSE54107.

Teratoma formation assay. 5×10^6 iPS cells were injected under the kidney capsules of 7-week-old severe combined immunodeficient (SCID) mice (CB17/SCID, Charles River). After 3 weeks, the mice were euthanized and lesions were surgically removed and fixed in formol or in 4% paraformaldehyde for cryosections. Same procedure was employed with 5×10^5 iPS cells injection in testicles. Human teratoma formation was conducted as previously described³⁴. All animal procedures were performed in accordance with the institutional guidelines (French ceccapp project 01369.01).

Quantifications and Statistics. Comparison of the reprogramming efficiencies was performed on littermate embryos. At least two different batches of MEFs and reagents were used. Statistical analyses of mean and variance were performed with Prism 6 (GraphPad Software) and Student's *t*-test. The littermates belong to an experimental group determined by the genotyping.

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Author contributions

D.O. performed the experiments from Figs 3, 4 and 5. O.F. and L.B.-G. performed the human reprogramming experiments and the characterization of human iPS cells from Figs 1 and 2. E.K. and M.P. prepared intestinal epithelium for reprogramming experiments. S.M. performed teratoma experiments with mouse iPS cells for Fig. 2. B.D. and B.G. provided mice for MEF derivation. J.G. and H.D. performed reprogramming experiments. ID performed flow cytometry analyses from Figs 4 and 5. N.G. and J.-Y.S. performed histological analysis of mouse teratoma from Fig. 2. J.G. performed iPS cells characterization assays and provided F.L. with reagents.

F.L. performed experiments from Fig. 1–5. F.L., A.B. and P.M. designed the study and F.L. and P.M. wrote the manuscript.

Additional information

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Title: netrin-1 bipotent signalling controls both self-renewal and lineage commitment in embryonic stem cells.

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Abstract:

Embryonic stem cells (ESC) are characterized by their ability to self-renew and to differentiate into all lineages^{1,2}. However, even if the switch from self-renewal to lineage commitment is crucial for embryo development, the mechanisms regulating this cell fate decision remain unknown. Here we report that the neuronal guidance cue netrin-1³ and its receptors neogenin and UNC5B control this process in mouse ESC. We show that netrin-1 confers to ESC transcriptomic and epigenomic features reflecting an intermediate configuration between the naïve ICM and the late epiblast. In line with this, netrin-1 is specifically expressed in the E4.5 pluripotent compartment *in vivo* and controls ESC derivation. The established view in stem cell biology advocates that signalling molecules act as pro-self-renewal or pro-lineage commitment cues in a given cell type. In contrast, we reveal that netrin-1 is bipotent in mouse ESC, meaning that it exerts both pro-self-renewal and pro-lineage commitment activities depending on the receptor it engages with. Mechanistically, netrin-1 promotes self-renewal via neogenin by alleviating Mapk signalling and triggering the dynamin-dependent degradation of UNC5B. In contrast, in presence of UNC5B, netrin-1 primes ESC for germ layer segregation by exacerbating their responsiveness to FGF. Altogether, our work identifies a "bipotent signalling" machinery by which a unique signalling molecule controls the switch from self-renewal to differentiation in stem cells. Due to the fact that netrin-1 expression is detected in adult stem cells and upregulated in a fraction of human cancers, we speculate that this machinery might operate in other stem cell types to control organism homeostasis and be deregulated in pathological contexts.

Metastable mESC are maintained in culture with LIF and serum (referred hereafter as serum/LIF). Concomitant activation of the WNT pathway and suppression of Mapk signalling (described hereafter as 2i) *in vitro* induces a "ground" state of pluripotency⁴⁻⁶ resembling the E2.5 morula and E3.5 inner cell mass (ICM)⁷. In an attempt to identify other signalling pathways controlling pluripotency, we compared the transcriptomes of serum/LIF mESC supplemented with Mek1/2 (PD0325901=PD), GSK3 (CHIR99021=CHIR) or both inhibitors for 48 hours. Among the transcripts encoding signalling molecules responsive to both inhibitors, *netrin-1* is induced by GSK3 inhibition and 2i at both transcript and protein levels (Fig. 1a-b). Other members of the netrin family (*netrin 4*, *-5*, and *-G1* and *-G2*)⁸ are not regulated by PD and CHIR (Extended Data Fig. 1a). We showed that netrin-1 protects somatic and pluripotent cells from apoptosis⁹ but here we failed to detect significant differences in cell death occurrence between culture conditions (data not shown), suggesting additional functions for netrin-1. Public data consultation confirmed *netrin-1* induction in ground state mESC and showed similar increase in naïve-like human ESC (Extended Data Fig. 1b and data not shown)¹⁰. GSK3 inhibition, or canonical WNT pathway stimulation, stabilizes pluripotency by alleviating the repressive effect of TCF7L1 on the core pluripotency network¹¹. Because netrin-1 expression is released by GSK3 inhibition, we asked whether it constitutes a target of the WNT pathway. Knockout mESC for TCF7L1 and LEF1 (Zhang J. et al., manuscript in revision) identify TCF7L1 as a main repressor and LEF1 as a main activator of netrin-1 expression (Fig. 1c and Extended Data Fig. 1c). CHIR-mediated netrin-1 release is abolished in mESC lacking TCF7L1 (Fig. 1c and Extended Data Fig. 1d), and TCF7L1 is bound to netrin-1 promoter (Extended Data Fig. 1e)¹². Netrin-1 expression is stimulated by CHIR and recombinant WNT3a in mESC (Fig. 1d and Extended Data Fig. 1f), demonstrating its regulation by the WNT pathway. Interestingly, Nanog and

Esrrb also induce netrin-1 (Extended Data Fig. 1g), suggesting auto-regulatory loops linking netrin-1 and pluripotency factors.

Netrin-1 is a laminin-related molecule harbouring pleiotropic functions in nervous system development and adult pathologies^{3,13,14}. Most of netrin-1 function has been shown to occur by signalling through the receptors deleted in colorectal carcinoma (*Dcc*) and UNC5-homologs (*Unc5h*, i.e., *Unc5A*, *Unc5B*, *Unc5C*, *Unc5D*). Tessier-Lavigne and colleagues demonstrated that neogenin (NEO1) also constitutes an effective receptor for netrin-1¹⁵⁻¹⁷. In serum/LIF mESC, DCC is absent but UNC5B and NEO1 are detected (Fig. 1e and Extended Data Fig. 1h). 2i switch is accompanied with a strong UNC5B decrease (Fig. 1e) while epiblast-like cells (EpiLC) induction¹⁸ correlates with *Neol* repression and *Unc5B* upregulation (Fig. 1f).

Netrin-1/Oct4 in situ hybridization on mouse pre-implanting embryos shows a transient and confined *netrin-1* expression in the pluripotent E4.5 epiblast while no signal is detected at earlier stages (Fig. 1g and data not shown), in agreement with published resources and differently from other transcripts encoding signalling molecules¹⁹ (Fig. 1h and Extended Data Fig. 1i). Single-cell transcriptomic data reveal a significantly higher *netrin-1* expression in epiblastic blastomeres than in PrE ones in E4.5 embryos²⁰ (Extended Data Fig. 1j). In agreement with *in vitro* data, transcripts for the netrin-1 receptors *Unc5b* and *Neol* are detected in early mouse embryos (Extended Data Fig. 1k), *Neol* starts to be detected in the E4.5 epiblast while *Unc5b* is induced later on in the E5.5 primed epiblast (Fig. 1h).

To characterize netrin-1 dynamics, mESC were derived from *netrin-1 βgeo* knock-in mice²¹. We show that netrin-1 expression is heterogeneous with βgal activity detected in 8% of serum/LIF and 26% of 2i mESC cells (Fig. 1i-j). Consultation of single cell transcriptomic data²² gave similar results (Extended Data Fig. 2a) and reveals that the mean *netrin-1* expression level per cell is statistically higher in 2i (Fig. 1k). When seeded at clonal density,

most of netrin-1 expressing colonies (97%) displayed mixed β gal staining, suggesting a switch in netrin-1 expression during the process of colony formation (Fig. 2l-m). To trace netrin-1 promoter activation, we generated netrin-1-CreERT2/LSL-GFP mESC expressing CreERT2 under the control of a 2.7kb netrin-1 promoter (Fig. 1n)⁹. Cre-recombinase translocates to the nucleus in presence of 4'OH-tamoxifen (TAM) and excises a lox-stop-lox cassette, leading to heritable constitutive GFP expression. FACS analysis of netrin-1-CreERT2/LSL-GFP mESC continuously treated with TAM for 49 days shows a gradual increase in the percentage of GFP expressing cells (Fig. 1o-p). Such steady increase over passages is unlikely to be due to a growth advantage conferred by GFP, as TAM withdrawal at day18 halted further increase, but rather results from spontaneous netrin-1 promoter activation. Altogether, these data reveal that netrin-1 signalling is highly dynamic in mESC and mouse embryos. Netrin-1, a transcriptional target of the WNT pathway, is specifically detected in the E4.5 epiblast. The netrin-1 receptor neogenin is linked with pluripotency while UNC5B accompanies priming for differentiation.

We next assessed whether netrin-1 confers particular properties to stem cells. We generated mESC expressing various netrin-1 versions, wild-type (netrin-1) or point mutated on distinct residues known to be critical for the netrin-1/UNC5B¹⁴ (netrin-1^{UNC5BMUT}) or netrin-1/neogenin¹⁷ (netrin-1^{NEO1MUT}) interactions, as depicted in Figure 2a-b. We established control, netrin-1, netrin-1^{UNC5BMUT} and netrin-1^{NEO1MUT} monoclonal lines expressing the HA-tagged netrin-1 versions at similar protein levels (Extended Data Fig. 2b), comparable to the one reached in 2i (Extended Data Fig. 2c). Colony formation assay reveals that mESC expressing wild-type netrin-1 form a significantly higher proportion of wholly undifferentiated colonies than control cells (Fig. 2c) and the overall colony number is also increased (Extended Data Fig. 2d-e). Netrin-1 also confers strong resistance to differentiation following LIF withdrawal (Fig. 2d-e). Both mutant versions of netrin-1 failed at conferring

such properties, suggesting that the ligand requires interaction with both UNC5B and neogenin to exert its function (Fig. 2c-e). These phenotypes are abrogated in revertant mESC generated by netrin-1 transgene excision (Fig. 2f-g and Extended Data Fig. 2f). Similar effects are observed using C57/Bl6 mESC and induced pluripotent stem cells (Extended Data Fig. 2g and data not shown), showing that netrin-1 enhances pluripotent stem cells properties in different strains. We next examined whether netrin-1 contributes to mESC derivation from embryos. Starting from 49 blastocysts obtained through netrin-1^{+/ β geo} mice crosses²¹, we derived 21 mESC lines distributed as follows: 16^{+/+}, 2^{+/ β geo} and 3 ^{β geo/ β geo} lines (Fig. 2h). This abnormally skewed genotype frequency far deviates from the 1:2:1 expected ratio, and such poor efficiency in stabilizing mESC lines with reduced netrin-1 level suggests its contribution to optimal pluripotency capture.

RNA-seq was next performed on control and netrin-1 mESC grown in serum/LIF. Netrin-1 expression affects 434 genes (fold change > 1.5 or < -1.5 at adjusted pvalue <0,05), with induction of the pluripotency transcripts *Nanog*, *Esrrb*, *Klf2*, *Klf4*, *Zfp42*, *Nr0b1*, *Sox2* and concomitant repression of differentiation genes, especially from the endoderm lineage *Dab2*, *Gata6*, *Gata4* and *Amn* (Fig. 2i). Among the top induced genes, we noticed a significant proportion of sequences flanked by long terminal repeats (LTR) of endogenous retroviruses (*Zscan4*, *Tcstv1*, *Tcstv3*, *Zfp352*, *AF067061*) (Fig. 2i and Extended Data Fig. 2h)²³. However, these inductions do not reflect a global release of MERVL-regulated sequences, as shown using luciferase reporters (Extended Data Fig. 2i)²³. At the protein level, netrin-1 triggers ESRRB, NANOG and SOX2 upregulation and concomitant repression of c-MYC while both mutant versions fail at conferring this pattern (Fig. 2j). A similar profile is observed in C57/Bl6 mESC and lost in revertant cells (Extended Data Fig. 2j-k). Netrin-1 mESC are more homogeneous at the morphological (Extended Data Fig. 2l) and molecular levels for ESRRB and NANOG (Extended Data Fig. 2m) and harbour enhanced ESRRB

transcriptional activity²⁴ (Extended Data Fig. 2n). Next, functional annotation clustering of differentially expressed genes by gene ontology (GO) PANTHER (protein analysis through evolutionary relationships) analysis shows that genes affected are enriched for terms associated with "embryo development", "endoderm development", "regulation of MAPK cascade" and "stem cell differentiation" (Fig. 2k). Significant differences are apparent for genes related to cell proliferation, in agreement with accelerated growth of netrin-1 mESC without differences in cell cycles features (Extended Data Fig. 2o-p). Because similar GO enrichments are observed in 2i mESC⁵, we asked whether netrin-1 triggers the acquisition of ground state pluripotency. We compared the transcriptomes of control and netrin-1 mESC grown in serum/LIF to previously published datasets (see Methods)^{7,19}. While serum/LIF and control samples cluster together as expected, netrin-1 cells acquire significant similarities with 2i mESC, demonstrating that netrin-1 confers transcriptomic features partially overlapping with the ground state (Fig. 2l). However, striking differences exist between both transcriptomes. 2i mESC are characterized by a global repression of differentiation genes⁵ while ectodermal genes remain unaffected in netrin-1 mESC (Fig. 2m). Such findings are in agreement with elevated SOX2 levels in netrin-1 mESC known to protect cells from mesendodermal differentiation²⁵, and suggest that the properties conferred by netrin-1 signalling are not fully equivalent to the ground state.

Due to the enrichment for genes related to MAPK cascade (Fig. 2k), we asked whether netrin-1 signalling modulates mESC sensitivity to other extracellular cues. In netrin-1 mESC grown in serum/LIF, active β -catenin, phospho-STAT3, phospho-JNK and phospho-AKT levels remain similar to control cells but phospho-ERK1/2 is significantly reduced (Extended Data Fig. 2q). Deprivation/stimulation experiments with recombinant FGF4 and LIF show that netrin-1 mESC are poorly responsive to FGF, as shown by reduced p-ERK1/2 activation (Fig. 3a). In similar settings, LIF-mediated p-ERK1/2 level is not affected by netrin-1 (Fig.

3b). In contrast, netrin-1 mESC are highly sensitized to CHIR and Wnt3a stimulation, as shown by strong LEF1 and active β -catenin inductions (Fig. 3c and data not shown) and higher nuclear and cytoplasmic β -catenin levels (Fig. 3d). We next assessed the consequences of netrin-1 signalling activation on mESC epigenome. Netrin-1 mESC have enhanced activity of the naïve Oct4 distal (DE), Jarid2 and Prdm14 enhancers (Fig. 3e)²⁶, suggesting epigenome modifications. ChIP-seq reveals a global decrease of both H3K4me3 and H3K27me3 histone marks in netrin-1 mESC (global enrichment for H3K27me3 ctrl=3.729 versus netrin-1=2.12 and Fig. 3f-g for H3K4me3) even if enriched H3K4me3 is detected at Nanog and Sox2 loci (Fig. 3h), in agreement with their upregulation in netrin-1 mESC (Fig. 2i). H3K27me3 is also significantly reduced at bivalent domains in netrin-1 mESC (Fig. 3i-j). Global DNA hypomethylation has been reported in male 2i mESC²⁷, with reduced expression of the *de novo* methyltransferases *Dnmt3A*, *B* and *L*, mimicking the E2.5 morula and E3.5 ICM (Extended Data Fig. 3a-b)¹⁹. *Dnmt3A* and *B* are also repressed in netrin-1 mESC, but to a lower extent than 2i, and *Dnmt3L* remains steady, in a similar manner as the E4.5 epiblast (Extended Data Fig. 3a-b). Restricted representation bisulphite sequencing (RRBS) reveals that netrin-1 mESC show similar methylation levels to control cells (mean = 0.25 \pm 0.003 and 0.24 \pm 0.004 respectively), in striking contrast to 2i (Fig. 3k-l). Differentially methylated features analysis (control vs netrin-1) reveals a small reduction of methylation in netrin-1 mESC across the genome (4410 hypomethylated versus 347 hypermethylated features) (Fig. 3m) that is not specific to any genomic feature (data not shown), confirming that netrin-1 mESC are in a different configuration than 2i. Next, transcriptome comparison of netrin-1 mESC with *in vitro* pluripotent states and embryonic samples (see Methods) was performed (Fig. 3n)¹⁹. As previously reported, 2i mESC cluster with E3.5 ICM and serum/LIF with E5.5 epiblast. In contrast, netrin-1 mESC transcriptome deviates from the E5.5 epiblast but does not cluster with E3.5 ICM and 2i mESC, suggesting an intermediate configuration, in

agreement with netrin-1 expression in E4.5 epiblast (Fig. 1g-h). This finding is further supported by the expression of key developmental genes. *Bmp4* and *Utf1* are low in 2i mESC and E2.5 morula while strong in netrin-1 mESC and E4.5 epiblast (Extended Data Fig. 3c-d). On the contrary, *Tcfcp2l1* and *Fbxo15* are strongly expressed in 2i mESC and early embryos but not in netrin-1 mESC and E4.5 epiblast (Extended Data Fig. 3c-d). Altogether, these data show that the sole expression of the netrin-1 ligand modifies mESC sensitivity to FGF and WNT signals, leading to the acquisition of unique functional, transcriptomic and epigenomic features, possibly reflecting an intermediate state between early ICM and late epiblast.

To dissect netrin-1 receptors function in mESC, we assessed UNC5B and neogenin expression during embryoid body (EB) differentiation. Similarly to EpiLC induction (Fig. 1f), EB formation is accompanied with NEO1 downregulation and UNC5B upregulation (Fig. 4a and Extended Data Fig. 4a), suggesting that the 2 receptors might harbour different functions, neogenin related to self-renewal and UNC5B to lineage commitment. Netrin-1 receptors have been shown to trigger opposite responses in response to netrin-1 in neurons, endothelial or immune cells^{28,29,30}. We therefore hypothesized that netrin-1 might harbour bipotent activities in mESC - pro self-renewal in neogenin^{high} settings and pro-lineage commitment in UNC5B^{high} ones. To examine neogenin and UNC5B respective function, stable knockdown mESC were established (Extended Data Fig. 4b-c). NEO1 knockdown mESC form a significantly reduced proportion of wholly undifferentiated colonies than control cells while UNC5B depletion has no significant effect in such settings (Fig. 4b). The overall colony number follows the same trend (Extended Data Fig. 4d-e). We next established mESC dox-inducible for neogenin in low (control NEO1-doxi) or high (netrin-1 NEO1-doxi) netrin-1 settings. Mechanistically, neogenin induction triggers phospho-ERK1/2 reduction in presence of high netrin-1 level (Fig. 4c), in contrast to what has been shown for the netrin-1 receptor DCC. Altogether, these data suggest that the netrin-1/neogenin axis improves mESC self-

renewal by limiting Mapk signalling. Regarding UNC5B function, we found that the repression of differentiation genes in netrin-1 mESC is mainly dependent on netrin-1 interaction with UNC5B (Extended Data Fig. 4f). However, UNC5B level is severely reduced in netrin-1 mESC in both total and membrane fraction, and this decrease is far less pronounced in netrin-1^{UNC5BMUT} mESC (Fig. 4d-e). Combined with the steady *Unc5b* transcript level in these cells (Extended Data Fig. 4g), these data led us to hypothesize that netrin-1 interaction with UNC5B might trigger its degradation, limiting therefore lineage commitment. Inhibition of lysosomal degradation with chloroquine (QC) shows that both UNC5B and NEO1 receptors are regulated by this pathway (Extended Data Fig. 4h)³¹. However, dynasore treatment led to strong UNC5B accumulation while leaving NEO1 unaffected (Fig. 4f), suggesting that netrin-1 binding to UNC5B might trigger its dynamin-dependent degradation. To evaluate netrin-1/UNC5B signalling function in mESC, we established mESC dox-inducible for UNC5B in low (control UNC5B-doxi) or high (netrin-1 UNC5B-doxi) netrin-1 settings (Fig. 4g-j). UNC5B induction severely reduces mESC resistance to differentiation in presence of high netrin-1 level (Fig. 4g-h). In contrast to neogenin, UNC5B induction triggers p-ERK1/2 induction (Fig. 4i) and concomitant NANOG, SOX2 and ESRRB reduction (Extended Data Fig. 4i). Deprivation and stimulation experiments with FGF4 reveal that UNC5B exacerbates netrin-1 mESC sensitivity to FGF to a higher degree than control cells (Fig. 4j), demonstrating that netrin-1 pro-self-renewal function is switched to a pro-lineage commitment one in presence of UNC5B.

We next assess whether the netrin-1/UNC5B/neogenin equilibrium controls differentiation. Sustained netrin-1 expression impairs the mESC differentiation potential both *in vivo* and *in vitro*. Teratomas derived from netrin-1 mESC are poorly differentiated (Fig. 4k) while EBs derived fail at repressing ESRRB, NANOG and SOX2 (Fig. 4l) and at inducing differentiation genes *Wnt3*, *Mixl1*, *Foxa2*, *Amn*, *Nes* and *Cdh2* (Fig. 4m). UNC5B induction is

also abrogated in EB derived from netrin-1 mESC (Fig. 4l), suggesting that its degradation by netrin-1 might impair lineage commitment. In line with this, netrin-1^{UNC5BMUT} mESC, in which netrin-1 is unable to degrade UNC5B, differentiate properly (Fig. 4l-m). Using netrin-1 UNC5B-doxi mESC, we show that UNC5B induction rescues partially netrin-1 mESC ability to downregulate pluripotency factors (Fig. 4n and Extended Data Fig. 4j). Altogether, these data show that the precise balance in the expression of netrin-1, neogenin and Unc5B tightly controls self-renewal and lineage commitment in mESC.

Loss of a pro-self-renewal signal in mESC leads to accelerated differentiation, as shown for LIF³², WNT³³ and BMP³⁴. In contrast, if netrin-1 function is bipotent, its depletion should negatively impact both mESC self-renewal and lineage commitment. To assess such hypothesis, we generated netrin-1 conditional knockout mESC (netrin-1^{fl/fl}) by crossing netrin-1^{fl/fl35} and Rosa26 CreERT2 mice. Because feeder cells secrete netrin-1, netrin-1^{fl/fl} mESC were adapted onto gelatin surfaces and netrin-1 depletion confirmed after Tam treatment (Extended Data Fig. 4k). Such depletion led to a transient downregulation of NANOG and SOX2 (data not shown) but netrin-1 depleted mESC can be stabilized and maintained in serum/LIF for >10 passages (data not shown). Colony formation assays performed with netrin-1^{fl/fl} mESC, treated or not with Tam, reveals that netrin-1 controls clonogenicity, in agreement with netrin-1 pro-self-renewal function (Fig. 4o-p). However, when netrin-1 depletion is induced just prior to EB formation, differentiation is not accelerated but rather delayed, in agreement with a pro-lineage commitment function (Fig. 4q). *In vivo*, Tam-treated netrin-1^{fl/fl} mESC generate smaller-size teratomas (Extended Data Fig. 4l) with reduced differentiation features (Fig. 4r) than untreated cells. Finally, netrin-1^{fl/fl} mESC injection into blastocysts give rise to color-coated chimera while Tam treatment abrogates such property (Fig. 4s-t). Altogether, these data support the hypothesis that netrin-1

function is bipotent in mESC and contributes to optimal mESC self-renewal and lineage commitment.

Self-renewal and lineage commitment are classically triggered by distinct signalling molecules in a given stem cell type. mESC self-renewal is sustained by LIF³², WNT³³ and BMP³⁴ while FGF is considered as the primary signal to exit self-renewal and acquire competence to differentiate in these cells³⁶. Interestingly in primed EpiSC, FGF and WNT triggers different responses, respectively inhibiting differentiation and promoting mesendodermal differentiation³⁷. A pro-differentiation function has been suggested for BMP4 during mESC differentiation but reflects a suppression of neural fate rather than an active mesodermal induction^{36,38}. In contrast to this established view, we describe a "bipotent signalling" machinery by which a unique signalling molecule exerts both pro-self-renewal and pro-lineage commitment activities, depending on the receptor engaged (Fig. 4u). Netrin-1 has been shown to mediate opposite responses during nervous system development - attracting or repelling axons - depending on the receptor engaged^{28 29}, highlighting the importance of the netrin-1 receptor repertoire for its function. In mESC, netrin-1 controls mESC self-renewal via neogenin while UNC5B converts netrin-1 pro-self-renewal function to a pro-lineage commitment one. We speculate that high UNC5B protein level might render netrin-1 incapable to degrade it, and stabilized netrin-1/UNC5B complexes will prime cells for lineage commitment by exacerbating their sensitivity to FGF. Despite such profound effect *in vitro*, mouse embryos lacking zygotic netrin-1 expression develop normally through the epiblast stage and die at embryonic day 14.5³⁹. Due to the bias in mESC derivation we observed in absence of netrin-1, a role in epiblast identity maintenance can not be ruled out. It remains to be investigated whether, as with gp130 stimulation, such role may be facultative rather than obligatory *in vivo*, and accentuated in the context of delayed implantation⁴⁰. Netrin-1 expression is not confined to mESC and is notably found to be expressed in other stem cells

(mesenchymal, neural) while upregulated in a fraction of human cancers¹⁴. It remains to be investigated whether bipotent signalling operates in other stem cell types and gets deregulated in pathological contexts.

Methods

Cell culture and derivation. *Netrin-1*^{βgeo} reporter²¹ and netrin-1 conditional knockout (*netrin-1*^{fl/fl})³⁵ mice were derived from C57/bl6 mixed background in N2B27/2i+LIF on feeders for 5 days and then transferred in serum/LIF. Cgr8 ES cells were cultured on gelatin in serum/LIF or KSR+LIF or in KSR 2i+LIF or N2B27 2i+LIF as previously described⁹. 293T and plat-E cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. PD0325901 (Millipore, 444968) and CHIR99021 (Millipore, 361571) were purchased from Merck Millipore. Luciferase assays were performed using dual luciferase reporter assay system (Promega E1910). MEF and HEK293 cells were transfected using Lipofectamine 2000 (Life technologies). Control, netrin-1, netrin-1^{UNC5BMUT} and netrin-1^{NEO1MUT} mESC were generated by stable transfection of Cgr8 mESC using FugeneHD reagent (promega E2691). Netrin-1 revertant mESC were obtained by treating mESC with 100 units TAT-CRE (SCR-508, Millipore) for 24h followed by FACS cell sorting of GFP expressing cells. Control NEO1-doxi, netrin-1 NEO1-doxi, control UNC5B-doxi and netrin-1 UNC5B-doxi mESC were generated by infecting control and netrin-1 mESC with dox-inducible plasmids. To assess p-ERK1/2 levels in response to receptors induction (Figure 4), cells are treated or not with Dox, grown in N2B27+dox media for 24h before collection for WB. Netrin-1 depletion in *netrin-1*^{fl/fl} mESC is induced by treatment with 4'OH-tamoxifen (Tam) at 0,2uM for 48 hours. The following inhibitors were used on mESC: Lyososome inhibitor Chloroquine (Sigma, C6628) 60uM 24h, Dynamin inhibitor Dynasore hydrate (Sigma, D7693) 60uM 24h. The following recombinant proteins were used as follows: Mouse

BMP4 (Peprotech, 315-27) 10ng/ml, Mouse Wnt3a (R&D Systems, 1324-WN) 50ng/ml, Human Fgf2 (Sigma, SRP-4037) 10ng/ml, Human Fgf4 (Peprotech, 100-31) 10ng/ml, Human Activin (Invitrogen, PHG9014).

Self-renewal and exit from pluripotency assays. For colony formation assay, mESC are plated at clonal density (60-100 cells per cm² depending on the strain) in serum/LIF. Media is changed every day during 7 days before detection of alkaline phosphatase positive colonies detection (AP0100-1KT, Sigma). For exit from pluripotency assays, mESC were grown for 7 days in serum minus LIF, then split and replated at clonal density (60-100 cells per cm²) for 7 additional days in serum/LIF.

Differentiation assays *in vitro* and *in vivo*. Serum deprivation/stimulation experiments assay is done by growing mESC overnight in N2B27 media without cytokines followed by exposure to the appropriate molecules for the times indicated in the figures. Control NEO1-doxi and netrin-1 NEO1-doxi mESC are grown in N2B27+dox media for 24h before collection and Embryoid body (EB) formation assay is realized by growing mESC in non adherent culture conditions in non-treated plastic plates for the indicated times. Epiblast-like (EpiLC) induction was performed as previously described¹⁸. Teratoma formation assay is performed by injecting 1X10⁶ mESC in the testis of 7-week-old severe combined immunodeficient (SCID) mice (CB17/SCID, Charles River). After 3-4 weeks, the mice were euthanized and lesions were surgically removed and fixed in formol or in 4% PFA for cryosections. For blastocyst injections, netrin-1^{fl/fl}, treated or not with Tam for 48h, were injected into BALB/cANRj blastocysts. The day before injection, frozen BALB/cANRj morulas from Quickblasto (JANVIER, France) were thawed according to the instruction given by the manufacturer and incubated overnight in KSOM medium (millipore, France) at 37°C, 5% CO₂. Between 5 and 15 cells were injected into expanded blastocysts in M2 medium (Sigma) using standard blastocyst injection techniques. Blastocysts were then

allowed to recover for a period of 1–3 h prior to being implanted into pseudo pregnant females. All animal procedures were performed in accordance with institutional guidelines (French ceccapp project 01369.01).

Constructs. To perform *in situ* hybridization on mouse embryos, a netrin-1 probe was cloned from mESC cDNA into pGEMTeasy (Promega) (sequence available on request). CreERT2 was subcloned from pMSCV-CreERT2-Puro (Addgene #22776) into pZsGreen1-DR vector to replace ZsGreen1-DR and a 2.7kb human netrin-1 promoter⁹ subcloned into this vector to generate netrin-1 CreERT2/LSL-GFP mESC. Point mutations^{14,17} were introduced into the pcagg-netrin-1-ires-puro vector to generate netrin-1 mutant versions using the quick site directed mutagenesis kit (Agilent). shRNA targeting the neogenin gene (CCCTCGAAACTCTCAAGATAT) is cloned in PLKO-TRC vector (Life technologies). shRNA targeting the Unc5b gene has already been described⁹. Neogenin and UNC5B Dox inducible pITR vectors are kind gifts from P. Mehlen.

Antibodies. Primary antibodies used in this study for immunoblotting and FACS are as follows: anti-OCT4 (Santa Cruz, sc5279), anti-NANOG (Cosmobio, RCA B000 2P-F), anti-SOX2 (Abcam, ab9759), anti-ESRRB (R&D Systems, PP-H6705-00), anti-c-MYC (Santa Cruz, sc-42), anti-Actin-hrp (Sigma, A3854), anti-LEF1 (Cell Signaling, C12A5), anti-NEO1 (Santa Cruz, sc6536), anti-UNC5B (Cell Signaling, D9M7Z), anti-Netrin-1 (R&D Systems, AF-6419), anti-phospho-ERK1/2 (Cell Signaling, T202, Y204-9101), anti-total ERK1/2 (Sigma, M5670), anti-phospho-STAT3 (Tyr705-D3A7), anti-total-STAT3 (Cell Signaling, 79D7), anti-phospho-AKT (Cell Signaling, S473-9271), anti-total AKT (Cell Signaling, 9272), anti-phospho-JNK (Santa Cruz, sc6254), anti-total-JNK (Cell Signaling, 9252), anti-p-38 (Cell Signaling, Thr180/Tyr182), anti-p-SMAD1/5/9 (Cell Signaling, D5B10), anti-GFP (CliniSciences, TP-401), anti-active- β -CATENIN (Millipore, 05-665), anti-TCF7L1 (kind gift from B. Merrill lab), anti-H3K27me3 (Diagenode #C15410195-10) and anti-H3K4me3

(Active Motif #61379). Membrane Fractionation is performed by using Mem-PER Plus Membrane Protein Extraction Kit (Thermofisher 89842). Nuclear and cytoplasmic extraction is performed by using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermofisher 78833).

Retroviral and lentiviral production. Plat-E cells were used to produce retroviral UNC5B dox inducible particles. 293T cells were used to produce lentiviral pLKO derived particles, as previously reported⁹.

Deep-sequencing. RNA quality was analysed using a Bioanalyser (Agilent). Libraries were constructed and sequenced on an illumina Hiseq 2000 by the Beckman Coulter Genomics and Genewiz companies. ChIP-seq experiments were performed as previously described²⁶. For ChIP-seq analysis, BWA was used for alignment of data to the mm9 genome with de-duplication performed using Picard tools, followed by peak calling using macs2 with narrow peak settings. To compare between control and netrin-1 conditions, homer annotatePeaks.pl was used with size = 2000, hist = 10 and -ghist used to generate read enrichments from both the control and netrin-1 sample using the control macs2 peaks. Data was then plotted in R using custom scripts. For analysis of RRBS data, UCSC liftOver was used to convert coordinates from mm10 to mm9 and the two replicates for control and netrin-1 samples were averaged at matching CpGs. For comparison to 2i conditions, WGBS data from GSM1027572 was used. Control, netrin-1 and 2i data were then merged so that only matched CpGs with coverage of at least 5X (for control and netrin-1) and 1X (2i) were used. Violinplots were generated in R using the library 'Vioplot' V0.2 and heatmaps were made using custom scripts in R. BedTools V2.25.0 was used to intersect CpGs with the following genomic features: high CpG promoters (HCP), CpG islands (CGI), low CpG promoters (LCP), CGI shores, exons, introns, long interspersed nuclear elements (LINE), short interspersed nuclear elements (SINE), long terminal repeats (LTR) and intracisternal A-particle elements (IAP), with all

annotation downloaded from UCSC with the exception of CGI and LCP/HCP which were computationally assigned. For any CpGs located within these features, mean methylation was calculated and plotted in R.

Hierarchical clustering. Control and netrin-1 mESC datasets were processed as follows. The “primary assembly” *Mus musculus* genome sequence (release GRCm38.p5) and transcriptome annotations (Ensembl release 87) were downloaded from the GENCODE website. Raw read data (fastq files) were mapped to these sequences using STAR (with parameters `--outFilterType BySJout`, `--outSAMtype BAM Unsorted`, and `--quantMode GeneCounts`). This last parameter allows direct conversion of the mappings into gene counts. These gene counts were transformed into FPKM and combined with the table provided by ref. 7. Only gene names present in both datasets were kept. The following process was applied: 1. keep genes with at least an average FPKM of 10 in at least one cell type (resulting in, as reported, 9639 genes); 2. normalize between datasets by subtracting, for each gene, the average $\log_2(\text{FPKM} + 1)$ in each dataset from this gene $\log_2(\text{FPKM} + 1)$ in the corresponding samples (geometric mean); 3. compute a dissimilarity matrix between samples by using the 1 – Spearman correlation between samples; 4. generate a hierarchical clustering using the “average” agglomeration method.

FACS. Analysis was performed on a BD LSRFortessa. Sorting was performed on a BD FACSDiVa. Cells were sorted, washed immediately and centrifuged before being plated directly in fresh medium or frozen for RNA extraction and gene expression analysis.

Quantifications and Statistics. Statistical analyses of mean and variance were performed with Prism 6 (GraphPad Software) and Student's t-test.

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Author contributions:

DO performed most of the experiments from figures 1-4 and contributed to the experimental design. AH, GF, PW and NC performed experiments. FL designed, supervised the study and wrote the manuscript. This work was supported by institutional grants from Inserm/Cnrs, Atip-avenir, Plan cancer, La ligue contre le cancer nationale et régionale (FL and PM),

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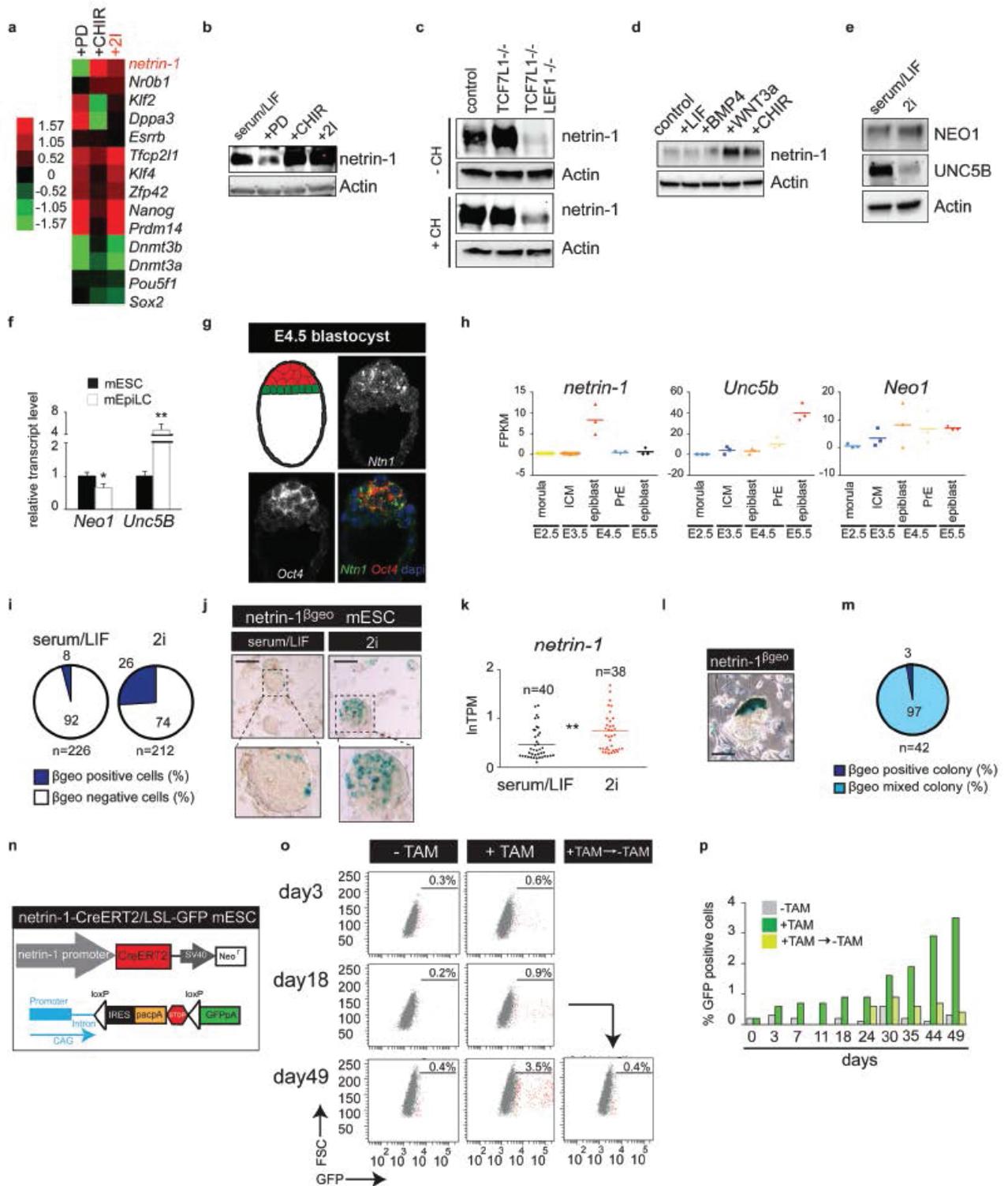


Figure 1. Netrin-1 signalling dynamics in pluripotent stem cells. **a**, Dendrogram depicts *netrin-1* and pluripotency factors transcript level in serum/LIF mESC supplemented with PD, CHIR or both (2i). Data are normalized to control mESC grown in serum/LIF. **b**, Western blot showing netrin-1 and Actin levels in similar settings. **c-d**, Netrin-1 is regulated by the WNT

pathway in mESC. **(c)** Netrin-1 and Actin western blot in control, TCF7L1^{-/-} and TCF7L1^{-/-} LEF1^{-/-} mESC treated (bottom panel) or not (top panel) with CHIR for 48 hours. **(d)** Western blot depicts netrin-1 level in mESC grown in serum/LIF treated for 24 hours with indicated molecules. **e-f**, neogenin and UNC5B expression in pluripotent cells and primed derivatives. **(e)** Neogenin and UNC5B protein level in serum/LIF and 2i mESC. **(f)** *Unc5b* and *neogenin* expression in Epiblast-like cells (EpiLC). Q-RTPCR data are expressed relative to mESC as the mean \pm s.d. (n=3-5). Student's t-test p<0,05 (*) or p<0,01 (**). **g**, *Netrin-1/Oct4 in situ* hybridization on E4.5 pre-implanting embryos. **h**, *Netrin-1*, *Unc5B* and *neogenin* expression during early mouse development. Data, extracted from ref. 19, present gene expression levels in fragments per kilobase of exon per million (FPKM) fragments mapped. Each dot corresponds to a replicate and the bar represents the average (n=3). **i**, Percentage of *netrin-1* β geo expressing mESC in serum/LIF or 2i mESC. n= total number of cells counted. **j**, Representative *netrin-1* β geo mESC colonies. Bars, 200 μ m. **k**, *Netrin-1* expression in single mESC. Data are extracted from ref. 22. n= number of cells analysed in each condition. Student's t-test p<0,01 (**). **l**, Representative *netrin-1* β geo mESC colony grown in serum/LIF. Bars, 200 μ m. **m**, Counting of colonies. n=number of colonies counted. **n**, Representation of the *netrin-1* CreERT2/LSL-GFP mESC. **o-p**, GFP expressing cells emergence in culture. **(o)** Representative FACS profile. Cells are grown in serum/LIF without or with 4'OH tamoxifen (TAM) for 49 days. TAM is withdrawn from media at day 18 (right panel). FACS profiles at day3, 18 and 49 for FSC-A and GFP. **(p)** Graph depicts the percentage of GFP expressing cells at various time points. Data corresponds to a single experiment, representative of three independent ones.

representative of three independent ones. **(e)** Colony counting. Data are the mean \pm s.d. (n=2). **f**, Strategy to generate netrin-1 revertant mESC. **g**, Exit from pluripotency assays. Similar settings as (d). **h**, The graph depicts the percentage of *netrin-1*^{+/+}, *+/βgeo* and *βgeo/βgeo* mESC lines obtained from *netrin-1*^{+/βgeo} heterozygous crosses. n=number of mESC lines generated. **i**, Volcano plot comparing the transcriptomes of control and netrin-1 mESC. Each dot corresponds to a transcript. Orange and green dots present a q value < 1. **j**, Western blot for ESRRB, NANOG, c-MYC, SOX2, OCT4. **k**, GO PANTHER analysis. **l**, Hierarchical clustering of control and netrin-1 transcriptomes with serum/LIF and 2i published datasets. Datasets from ref. 7 are used (see methods for details). **m**, Differentiation-related genes expression in control, 2i and netrin-1 mESC. Dendrogram presents Q-RTPCR data. Value 0 (log 1) is given to control mESC and data corresponds to the mean of two independent experiments. Color scale is provided.

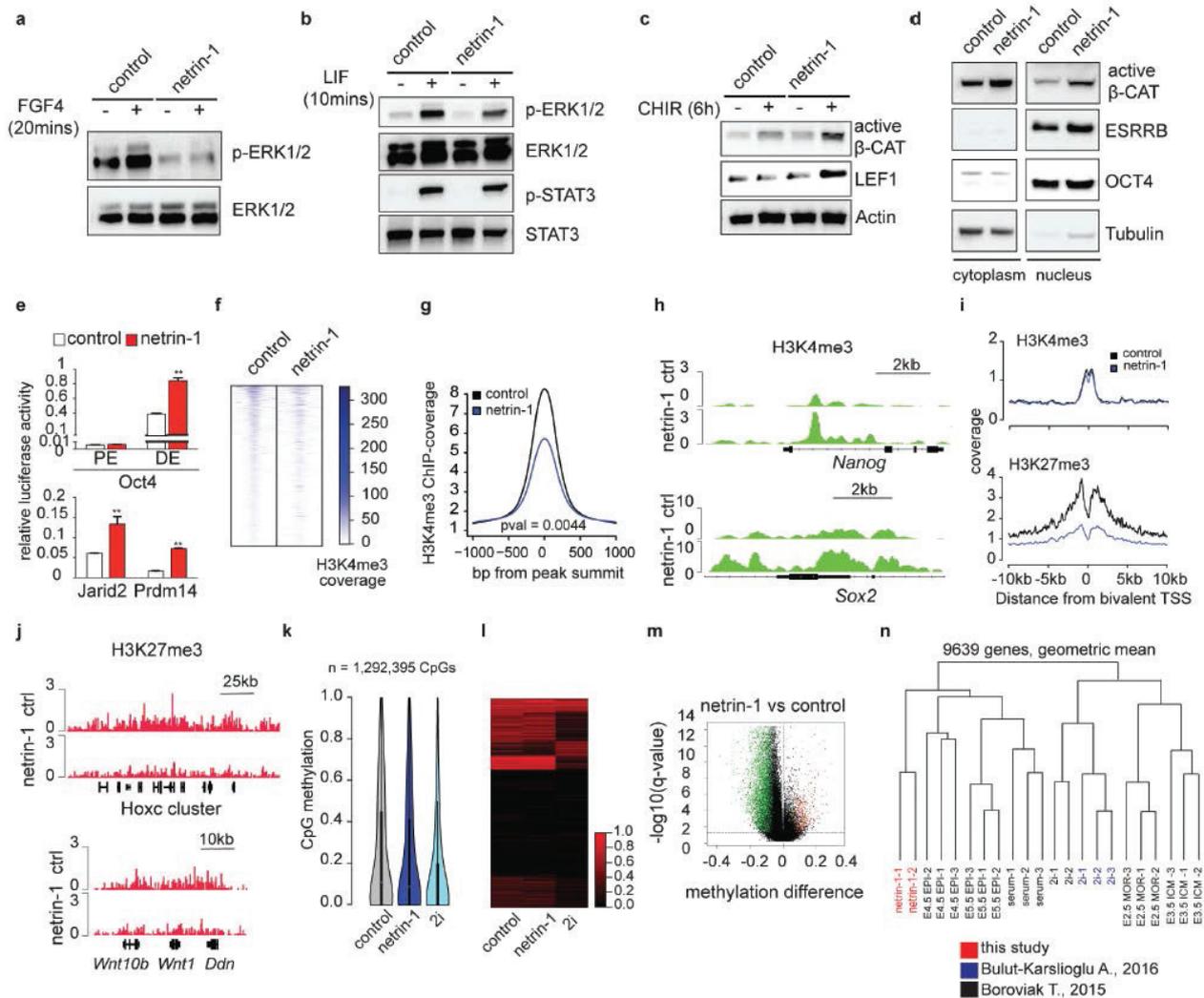


Figure 3. Netrin-1 impacts mESC epigenome. **a**, Western blot depicts total ERK1/2 and phospho-ERK1/2 (p-ERK1/2) levels in deprivation/stimulation with FGF4. **b**, Similar protocol as (a) with LIF and WB for total ERK1/2, phospho-ERK1/2 (p-ERK1/2), total STAT3 and phospho-STAT3 (p-STAT3). **c**, Similar protocol as (a) with CHIR and WB detection of active β -CATENIN and LEF1. **d**, Active β -CATENIN, ESRRB and OCT4 western blot in cytoplasm and nucleus enriched fractions of control and netrin-1 mESC. Similar protocol as (c). **e**, Specific enhancer activity characterizes netrin-1 mESC. Data are normalized to Renilla activity and expressed as the mean \pm s.d. (n=3). Student's t-test $p < 0,01$ (**). **f-g**, H3K4Me3 distribution in control) and netrin-1 mESC. (**f**) Heatmap displaying the H3K4me3 ChIP read enrichment for control and netrin-1 mESC for regions defined as

H3K4me3 peaks in control cells. For every peak, the summit (at 0) +/- 1000 bp is shown. Only peaks with q-value < 0.01 and mean enrichment for the region in the top third quantile are displayed ($n = 11,131$). The read enrichment is described by the color key to the right. **(g)** For all significant (qvalue < 0.01) H3K4me3 peaks in control cells ($n = 44,486$), the H3K4me3 ChIP read enrichment is displayed for control and netrin-1 mESC. ($p = 0.0044$; t.test). **h**, Representative browser shots of H3K4me3 enrichment at Nanog and Sox2 loci. **i**, H3K27Me3 enrichment at bivalent domains in control and netrin-1 mESC. **j**, Representative browser shots of H3K27me3 enrichment at bivalent loci. **k**, Violin plots display methylation levels for control, netrin-1 and 2i mESC for 1.3M matched CpGs. **l**, Heatmap displays methylation levels for 1.3M matched CpGs where each horizontal line is one CpG. **m**, Volcano plot showing features that are differentially methylated between control and netrin-1 mESC. Significantly hypomethylated (green dots) and hypermethylated (red dots) are represented. **n**, Hierarchical clustering of netrin-1 mESC transcriptome with serum and 2i mESC alongside embryonic samples published datasets. Datasets from ref. 7 were used (see methods for details).

Ozmadenci D. et al., Figure 4

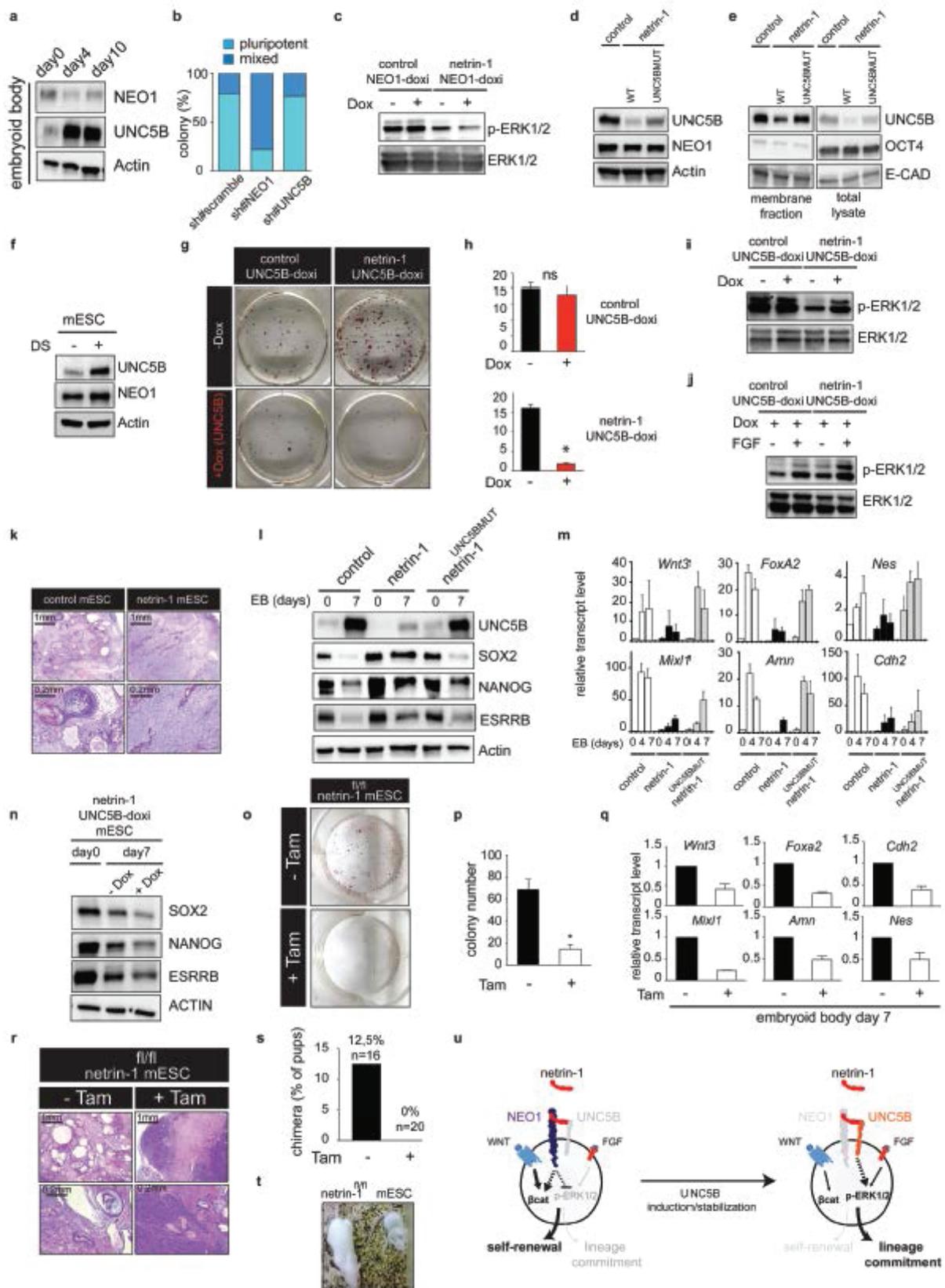
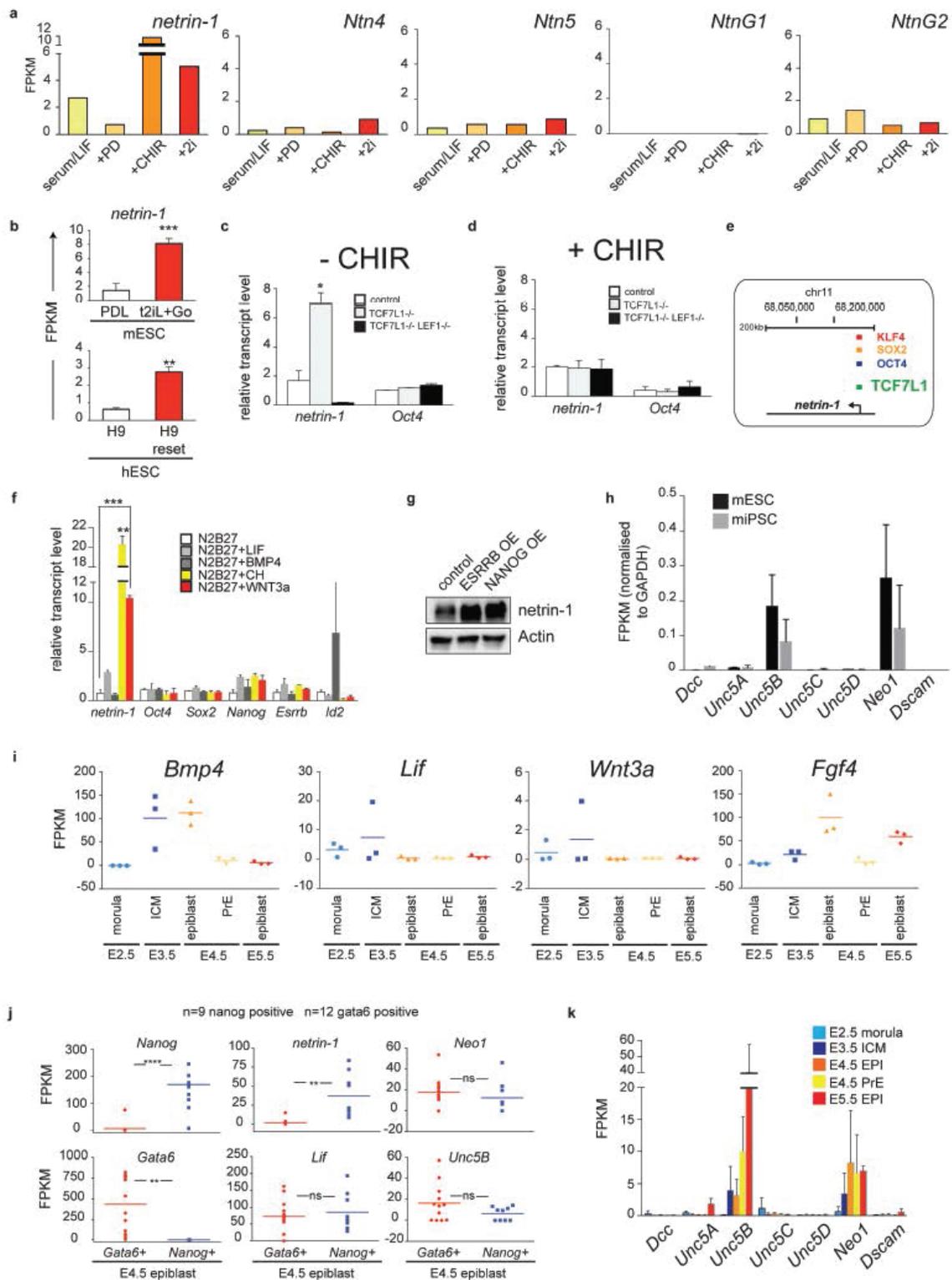


Figure 4. Netrin-1 exerts bipotent activities in mESC. **a**, Western blot for neogenin and UNC5B is performed after 4 and 10 days of EB formation. **b**, Quantification of colony

formation assay. Wholly (pluripotent) and partially (mixed) undifferentiated colonies are scored. Graph shows a single experiment, representative of two independent ones. **c**, Neogenin reduces phospho-ERK1/2 levels in presence of high netrin-1 level. Western blot depicts total ERK1/2 and phospho-ERK1/2 (p-ERK1/2) levels. **d**, Western blot for neogenin and UNC5B in control, netrin-1 and netrin-1^{UNC5BMUT} mESC. **e**, Western blot analysis of isolated membrane proteins and total cell lysates of control, netrin-1 and netrin-1^{UNC5BMUT} mESC. **f**, Western blot following dynamin inhibitor treatment of mESC. **g-h**, Exit from pluripotency assays are performed with control UNC5B-doxi and netrin-1 UNC5B-doxi mESC treated or not with doxycycline. **(g)** Representative pictures of a single experiment representative of two independent ones. **(h)** Colony counting. Data are the mean \pm s.d. (n=2). Student's t-test $p < 0,05$ (*). **i**, UNC5B induces phospho-ERK1/2 in presence of high netrin-1 level. Western blot depicts total ERK1/2 and phospho-ERK1/2 (p-ERK1/2) levels. **j**, UNC5B exacerbates mESC sensitivity to FGF in presence of high netrin-1 level. WB depicts total ERK1/2 and phospho-ERK1/2 (p-ERK1/2) levels. **k**, Teratoma histological analysis of control and netrin-1 mESC. 2 different magnifications are presented. **l-m**, Embryoid body formation of control, netrin-1 and netrin-1^{UNC5BMUT} mESC. **(l)** UNC5B, ESRRB, SOX2 and NANOG western blot in mESC and day7 EBs. **(m)** Q-RTPCR depicts mesoderm (*Wnt3a* and *Mixl1*), endoderm (*FoxA2* and *Amn*) and ectoderm (*Nes* and *Cdh2*) transcript level in mESC and EBs at day 4 and day7. Data are the mean \pm s.d. (n=3). **n**, Western blot depicts ESRRB, SOX2 and NANOG expression in 7 days EB treated or not with Dox. **o-p**, Colony formation assay performed with netrin-1^{fl/fl} mESC. **(o)** Representative pictures and **(p)** colony countings. Data are the mean \pm s.d. (n=2). Student's t-test $p < 0,05$ (*). **q**, Embryoid body formation of netrin-1^{fl/fl}. QRTPCR depicts similar transcript levels as (m) in netrin-1^{fl/fl}, treated or not with Tam, and induced to form EB for 7 days. Data are the mean \pm s.d. (n=2). **r**, Teratoma histological analysis. 2 different magnifications are presented. **s**, Blastocyst injections, percentage of

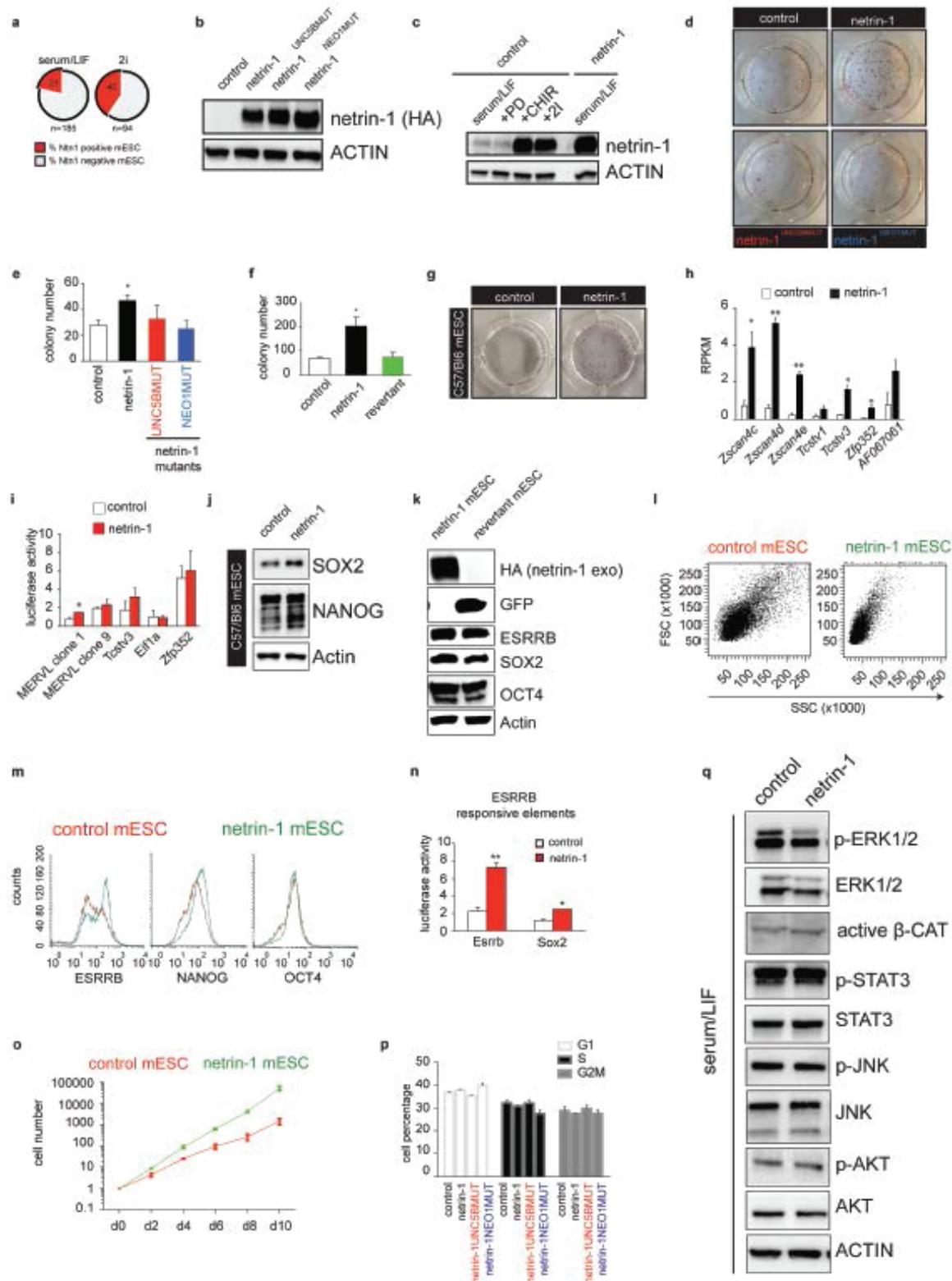
chimera obtained are shown, n=number of live pups obtained. **t**, Example of color-coated chimera obtained following injection of netrin-1^{fl/fl} mESC. **u**, Graphical summary of the results.



Extended data Figure 1. Netrin-1 signalling dynamics in pluripotent stem cells. a, Data present fragments per kilobase of exon per million fragments mapped (FPKM) values for *netrin-1*, *Ntn4*, *Ntn5*, *NtnG1* and *NtnG2* in serum/LIF mESC treated or not with PD, CHIR or

both. **b**, *Netrin-1* transcript level in mouse ground state and human naïve-like pluripotent stem cells. Data are extracted from ref. 10 corresponding to RNA-seq data. Data are the mean \pm s.d. (n=3). Student's t-test $p < 0,01$ (**) or $p < 0,001$ (***). **c-d**, *Netrin-1* and *Oct4* transcripts level in control, *TCF7L1*^{-/-} and *TCF7L1*^{-/-} *LEF1*^{-/-} mESC treated (d) or not (c) with CHIR for 48 hours. Q-RTPCR data are expressed relative to mESC as the mean \pm s.d. (n=3). Student's t-test $p < 0,05$ (*). **(e)** *TCF7L1* is bound to *netrin-1* promoter in mESC. *TCF7L1*, *OCT4*, *SOX2* and *KLF4* ChIP-seq data were extracted from ref. 12. **f**, Q-RTPCR depicts *netrin-1*, *Oct4*, *Sox2*, *Nanog*, *Esrrb* and *Id2* expression in mESC grown in serum/LIF. Q-RTPCR data are expressed relative to mESC as the mean \pm s.d. (n=3). Student's t-test $p < 0,05$ (*), $p < 0,01$ (**) and $p < 0,001$ (***). **g**, Western blot shows *netrin-1* level in mESC stably transfected with *Esrrb* (*Esrrb* OE) or *Nanog* (*Nanog* OE) expression constructs. **h**, RNA-seq depicts *Dcc*, *Unc5a*, *Unc5b*, *Unc5c*, *Unc5d*, *Neol* and *Dscam* transcripts level in mouse ES and iPS cells. FPKM values are normalized to *gapdh* and are expressed as the mean \pm s.d. (n=2). **i**, Data, extracted from ref. 19, present *Bmp4*, *Lif*, *Wnt3a* and *Fgf4* transcripts level in FPKM. Each dot correspond to a replicate and the bar represents the average (n=3). **j**, *Netrin-1*, *Nanog*, *Gata6*, *Lif*, *Neol* and *Unc5b* expression in single blastomeres. Data are extracted from ref. 20. Data correspond to FPKM values for 10 *nanog* positive cells and 15 *gata6* positive blastomeres. Each dot corresponds to a cell, the bar is the mean \pm s.d. **k**, Graph depicts *netrin-1* receptors transcripts level in different embryonic stages. Data, extracted from ref. 19, present gene expression levels in FPKM. Data are the mean \pm s.d. (n=3).

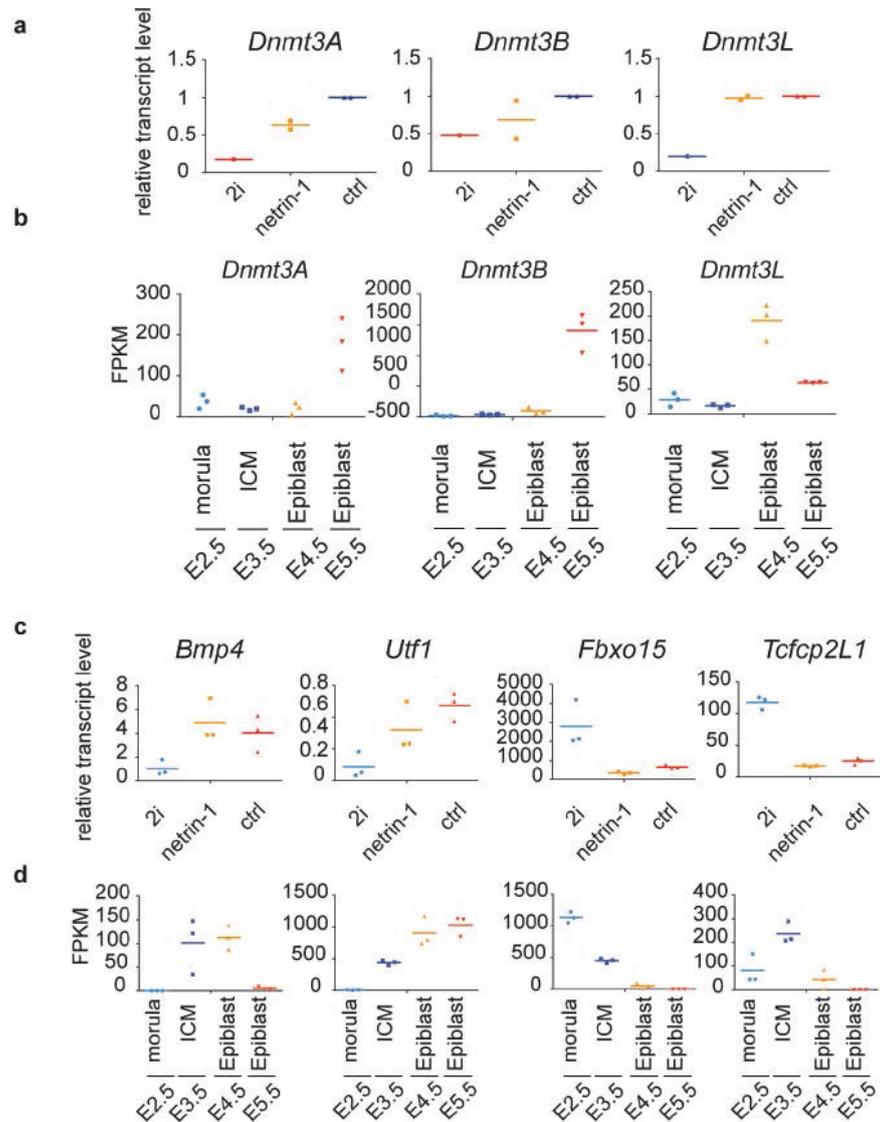
Ozmadenci D. et al., Extended Data Figure 2



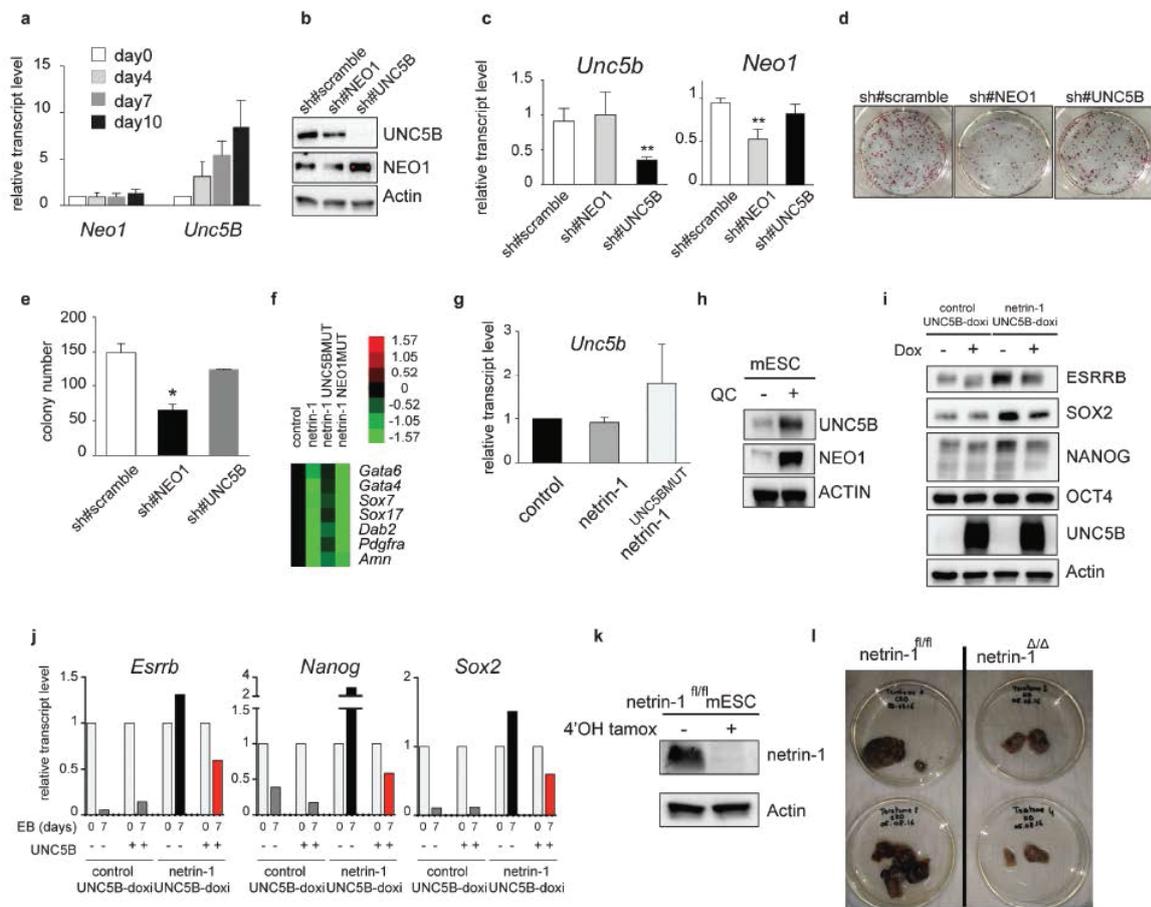
Extended Data Figure 2. Netrin-1 confers specific pluripotency traits to mESC. a, *Netrin-1* expression in single mESC in Serum/LIF and 2i. Single-cell transcriptomic data are extracted from ref. 22. n=number of cells analysed in each condition. Data present the

percentage of cells expressing *netrin-1*. **b**, Western blot depicts exogenous netrin-1 (HA) and Actin levels in control, netrin-1, netrin-1^{UNC5BMUT} and netrin-1^{NEO1MUT} mESC. **c**, Western blot depicts netrin-1 and Actin levels in control mESC supplemented with PD, CHIR or 2i alongside netrin-1 mESC grown in serum/LIF. **d-e**, Quantification of colony formation assay. **(d)** Representative pictures. **(e)** Overall colony counting. Data are the mean \pm s.d. (n=2). **f**, Characterization of netrin-1 revertant mESC. Same as (e) with control, netrin-1 and revertant mESC. **g**, Quantification of colony formation assay. Control and netrin-1 expressing mESC were established from C57/Bl6 mESC and colony formation assay performed. Representative pictures. **h**, *Zscan4c*, *Zscan4d*, *Zscan4e*, *Tcstv1*, *Tcstv3*, *Zfp352* and *AF067061* transcript level in control and netrin-1 mESC. FPKM values are expressed as the mean \pm s.d. (n=2). **i**, The activity of MERVL-regulated sequences is similar in control and netrin-1 mESC. MERVL-regulated constructs are transfected in control and netrin-1 mESC. Data are normalized to Renilla activity and expressed as the mean \pm s.d. (n=3). **j**, Western blot depicts NANOG and SOX2 expression in cells from (g). **k**, Western blot depicts exogenous netrin-1 (HA), GFP, ESRRB, SOX2, OCT4 and Actin levels in netrin-1 and revertant mESC. **l**, Representative FSC and SSC FACS profile for control and netrin-1 mESC. **m**, ESRRB, NANOG and OCT4 distribution in control and netrin-1 mESC. **n**, ESRRB transcriptional activity is enhanced in netrin-1 mESC. Data are normalized to Renilla activity and expressed as the mean \pm s.d. (n=3). Student's t-test $p < 0,05$ (*) or $p < 0,01$ (**). **o**, Control and netrin-1 mESC proliferation curves. Data are the mean of 2 independent experiments. **p**, Cell cycle features of control and netrin-1 mESC. **q**, Western blot depicting total ERK1/2 and phospho-ERK1/2 (p-ERK1/2), active β -CATENIN, total STAT3 and phospho-STAT3 (p-STAT3), total JNK and phospho-JNK (p-JNK), total AKT and phospho-AKT (p-AKT) and Actin levels in control and netrin-1 mESC.

Ozmadenci D. et al., Extended Data Figure 3



Extended Data Figure 3. Netrin-1 impacts mESC epigenome. **a**, *Dnmt3A*, *3B* and *3L* transcript levels in 2i, netrin-1 and control (serum/LIF) mESC. Data corresponds to Q-RTPCR results for 2 independent experiments with value 1 given to the level in control cells. **b**, *Dnmt3A*, *3B* and *3L* transcript levels in different embryonic stages. Data are extracted from ref. 19. **c-d**, *Bmp4*, *Utf1*, *Fbxo15* and *Tcfcp2L1* expression levels in netrin-1, serum and 2i mESC and during embryonic development. *In vitro* data (c) corresponds to Q-RTPCR experiments while *in vivo* data (d) are extracted from ref. 19.



Extended Data Figure 4. Netrin-1 exerts bipotent activities in mESC. **a**, QRTPCR depicts *Neo1* and *Unc5b* level at day0, day4, day7 and day10 of EB formation. Data are the mean \pm s.d. (n=3). **(b)** Western blot for NEO1, UNC5B and Actin in sh#scramble, sh#NEO1 and sh#UNC5B mESC polyclonal lines. **(c)** Transcript level. Data are the mean \pm s.d. (n=3). **d-e**, Overall colony number in colony formation assay. **(d)** Representative pictures. **(e)** Countings. Data are the mean \pm s.d. (n=2). **f**, Differentiation associated genes expression in control, netrin-1, netrin-1^{UNC5BMUT} and netrin-1^{NEO1MUT} mESC. Dendrogram presents RNA-seq data with value 0 (log 1) given to control mESC. Color scale is provided. **g**, QRTPCR depicts *Unc5b* transcript level in control, netrin-1 and netrin-1^{UNC5BMUT} mESC. Data are the mean \pm s.d. (n=3). **h**, UNC5B and NEO1 expression detected by western blot following lysosome inhibitor treatment (60uM Chloroquine for 24h) **i**, Western blot of ESRRB, NANOG, SOX2, OCT4, UNC5B and Actin in control UNC5B-doxi and netrin-1 UNC5B-doxi mESC treated

or not with Dox. **j**, QRTPCR depicts *Esrrb*, *Nanog* and *Sox2* transcript level in control UNC5B-doxi and netrin-1 UNC5B-doxi mESC treated or not with Dox at day0 and day7 of EB formation. Data are normalized to day0 control mESC and are the mean \pm s.d. (n=2). **k**, Western blot depicts netrin-1 and Actin levels in netrin-1^{fl/fl} mESC treated or not with 4'OH-tamoxifen for 3 days before collection. **l**, Pictures of teratoma obtained following injection of netrin-1^{fl/fl} and netrin-1 ^{Δ/Δ} mESC.

DISCUSSION

Section-1: New Discoveries and Hypotheses

Identification of new roles for Netrin-1 signaling in reprogramming and in PSC

Prior to my thesis, Netrin-1 had not been known to be, or suspected to be a regulator of pluripotent stem cells (PSC). During my thesis, the work we performed enabled the unequivocal identification of Netrin-1 as a novel regulator of reprogramming and pluripotency (**Figure-17**).

Netrin-1 signaling regulates reprogramming

The discovery of iPSC generation in 2006 was considered so groundbreaking that it brought the Nobel Prize to Shinya Yamanaka in 2012. The iPSCs generation represents the translational medicine. Yet, somatic cell reprogramming is a very inefficient process, which frequently results in undesired cell fates such as senescence or apoptosis. It is clear that novel approaches that increase the efficiency of this process could have significant future impact.

We identified Netrin-1 as a regulator of the reprogramming process. Moreover, we showed for the first time that the dependence receptors act as negative regulators of the early phase of reprogramming and pluripotency maintenance. In our studies, we further identified that Netrin-1 exhibits pleiotropic activities within these systems. These activities include cell survival. In this case, the results were consistent with the Dependence Receptor hypothesis, effectively blocking dependence receptor apoptotic-activity via binding to these receptors. The results are strengthened by the fact that the dependence receptors DCC and UNC5B were identified in the same screen as potential apoptotic regulators and interactors of Netrin-1.

We further demonstrated that utilization of recombinant Netrin-1 improved the iPSCs generation process by at least one mechanism, which is inhibition of dependence receptor 'DCC' apoptotic activity. This discovery justifies the improvement of iPSC generation process in the presence of Netrin-1. However, given our other results, it is likely that Netrin-1 guides further functions during this process. These hypotheses will be discussed in detail below.

Netrin-1 signaling regulates pluripotency and lineage commitment

Pluripotency is a transient state during early embryogenesis. Highly dynamic, and distinct, pluripotency states with unique molecular and functional features exist *in-vivo*. It is now appreciated that these states can be captured *in-vitro*, and that the differences between the states may have important implications for the maintenance and use of these cells. However, the regulatory signaling pathways that define these pluripotency states, enabling transitions between them, and exit from pluripotency, are poorly understood. According to the classical view, in a given cell, the factors that govern pluripotency, and lineage commitment, are distinct. This approach identifies a set of stem cell specific factors, but would not identify potentially important regulatory mechanisms that use factors common to both processes. As we demonstrated, common factors can play distinct roles in stem cells.

Here, we identified that a same protein is able to guide different cell fates (**Figure-17**). We showed for the first that '*the one who guides*' -Netrin-1-stimulates remarkably opposite effects by promoting both self-renewal of mESC, and also acting in a critical role in lineage commitment. This dichotomy is reminiscent of the well-characterized capacity of Netrin-1 to mediate opposed cellular responses during nervous system development. Our findings demonstrate a unique and unexpected conservation of highly context-dependent Netrin-1 signaling during early embryogenesis. This finding may contribute to change the approach to identify

factors that regulate stemness vs. differentiation. Broad genetic or transcriptional screens are frequently based on the uniqueness of one data set vs. another. By their design, these types of screens would miss Netrin-1 because of its expression during both processes.

We identify uniform sets of genes implicated in opposed process such as 'stemness and differentiation', 'survival and death', 'activation and repression' or 'enhancement and attenuation.' However, it may be prudent to consider the occurrence of more complex processes, such as the one governed collectively by Netrin-1. The problem that presents is how to identify proteins, like Netrin-1 with the ability to act pleiotropically. The clues to this may be in searching not only individual genes, but perhaps in using network approaches. These networks could include both ligands and receptors, and even critical signaling elements that influence the function of the receptor-ligand network. These searches could then provide testable hypothesis for continued investigation.

The bipotent signaling response of Netrin-1 is mediated via the differential expression and actions of its receptors. Netrin-1 signaling improves self-renewal of mESC and promotes naïve pluripotency at least by three mechanisms. These are (1) inhibition of UNC5B-induced apoptosis, (2) inhibition of UNC5B induced-pro-commitment signals via Netrin-1/UNC5B interaction and subsequent UNC5B degradation and (3) triggering Netrin-1/NEO-1-induced-self renewal activity. Moreover, in mESCs, 'strongly induced'- Netrin-1 receptor UN5CB enables a transition from naïve pluripotency (in mESC) to an intermediate state (in mEpiLC), which is a preparatory state to lineage priming. This prepares the cells for exit from pluripotency. Thus, lineage commitment (in EB) occurs coincident with strongly induced UN5B, in a Netrin-1 dependent manner. These results are supported by the results *in-vivo*. Netrin-1 knockout cells are retarded in their capacity to engage all cell lineages.

Aside from differential receptor expression, the bifunctional Netrin-1 signaling

response in nervous system includes the implication of several extracellular and intracellular factors. The factors acting extracellularly or at the cell surface are heparan sulfate, and G protein-coupled receptors, while Ca^{2+} , cAMP, PKC, PKA, and Rho act intracellularly. Identifying additional factors implicated in Netrin-1 guided differential responses in stem cells will be of benefit for understanding the Netrin-1-guided cell fates changes.

Dynamic regulation of Netrin-1 gene by multiple factors during reprogramming and in PSCs

We identified that during the initiation of reprogramming; Klf4 and Oct4 transgenes as well as a NURD complex composed of Mbd3, Mta1, Chd4 repress the Netrin-1 promoter. This leads to a transient decrease in its expression. In mESCs, Netrin-1 expression is a target of the WNT signaling pathway. Netrin-1 expression is activated by Lymphoid Enhancer Binding Factor-1 (Lef1) and repressed by Transcription Factor 7 like-1 (Tcf711 or Tcf3) **(Figure-18)**. These specific regulations of Netrin-1 expression may occur in different contexts. I would posit that identifying the regulators of differential Netrin-1 receptor expression in stem cells and in reprogramming could be extended to improve our understanding of receptor dynamics, which is an important mediator of Netrin-1 signaling response. This may help our understanding of other factors leading to developmental process or to pathologies such as cancer, that are known to exhibit the deregulation of Netrin-1.

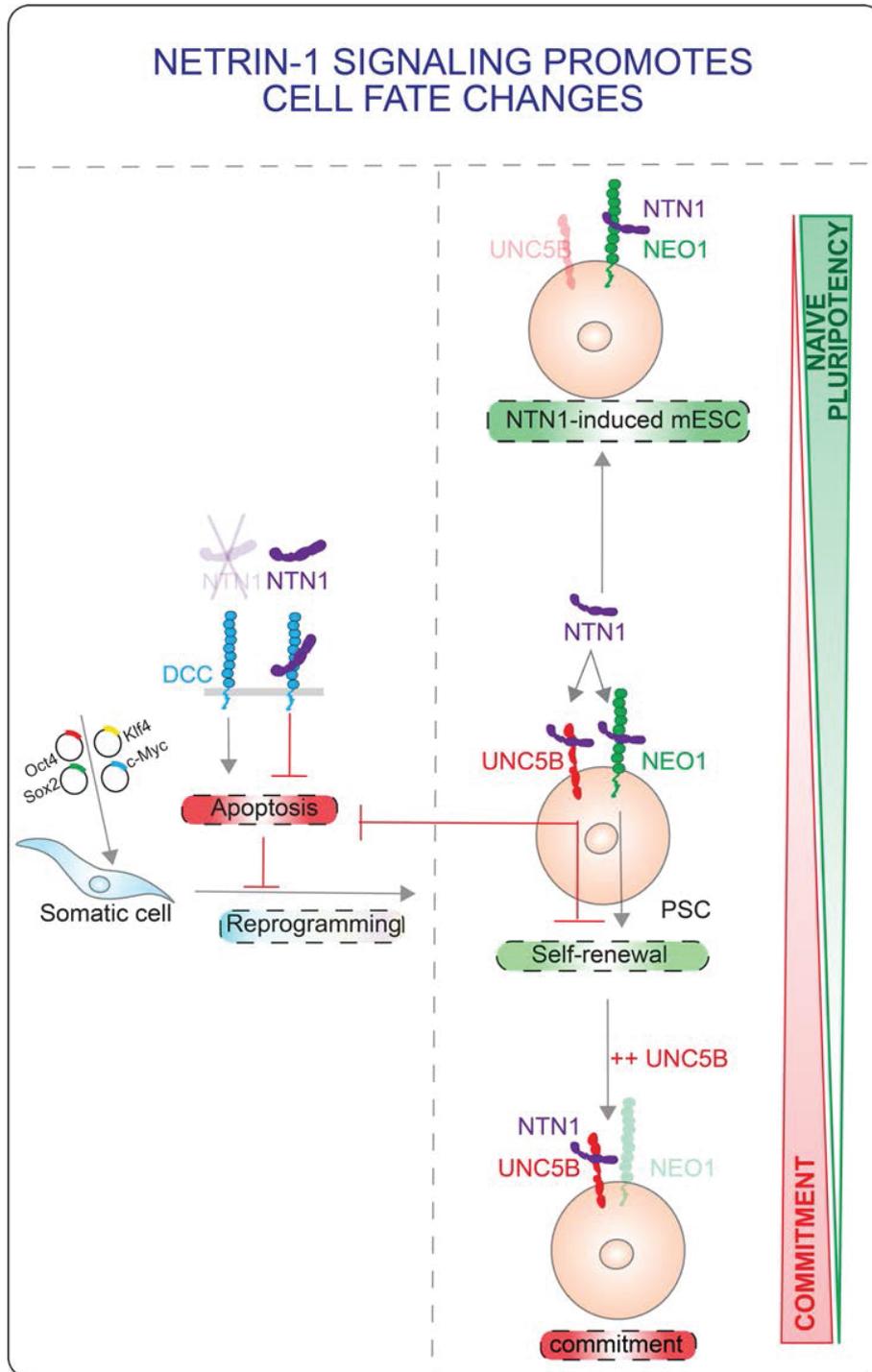


Figure-17: Netrin-1 (NTN1) signaling promotes cell fate changes.

During somatic cell reprogramming, Netrin-1 signaling improves reprogramming by blocking DCC apoptotic activity. In PSCs, Netrin-1 promotes naïve pluripotency by three mechanisms: (1) via inhibition of UNC5B- induced apoptosis, (2) via interaction with and degradation of UNC5B that induces pro-commitment activity, (3) via interaction with Neogenin (NEO1) that induces pro-self renewal activity. In PSC, Netrin-1 signaling enables transitions from naïve pluripotency (in mESC) to an intermediate state (in mEpiLC) and to the lineage commitment (in EB) via interaction with UN5B.

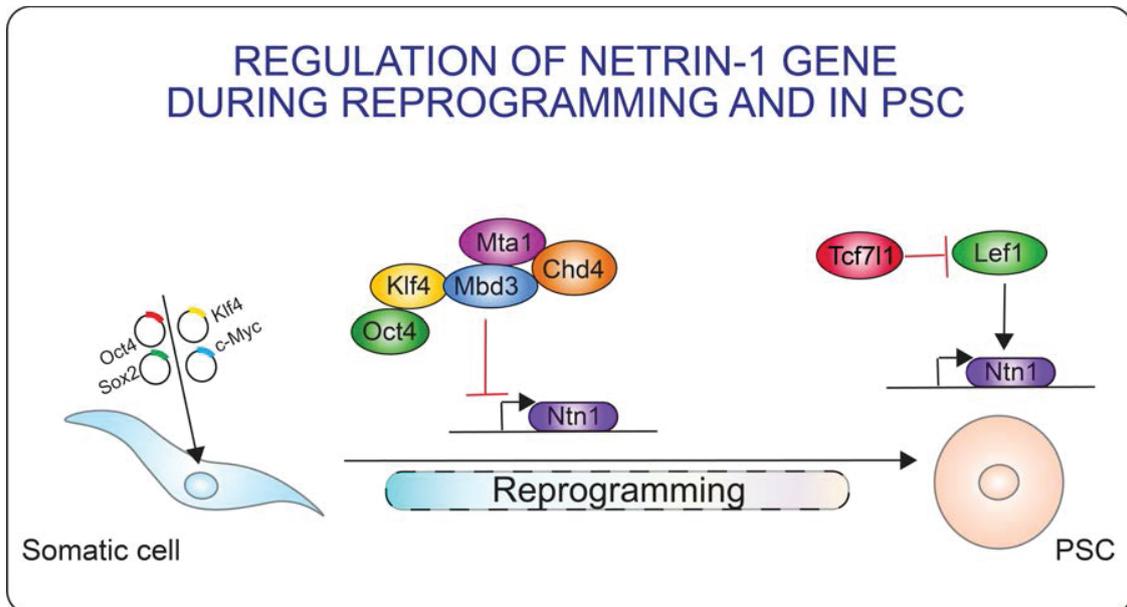


Figure-18: Netrin-1 expression is dynamically regulated during reprogramming and in PSC. During early phases of reprogramming, Klf4, Oct4 and a NURD complex composed of Mbd3, Chd4 and Mta1 repress Netrin-1. In PSCs, Netrin-1 is repressed by Tcf711 and activated by Lef1.

Section-2: Undetermined Questions and New Hypotheses

Could Netrin-1, a laminin-like protein, be a new extracellular matrix candidate protein to improve reprogramming and PSC self-renewal?

Physiologically, all cells exist in the milieu of the larger extracellular matrix. Laminin is an ECM component that forms the layers between normal tissues. It plays key roles in neurogenesis. Most importantly, it is used in stem cell culture, and one of its major receptors cD49f, is used as a stem cell marker. Netrin-1 is a small, laminin-like protein which, like laminin itself, has very poor solubility. Thus, it appears that Netrin-1 could be used, much like laminin, as an adhesion surface for stem cell culture.

Among the number of recent advances that have improved iPSC reprogramming, the use of extracellular matrices and the use of pre-coated-plates rendering iPSC generation more potent is noteworthy. Such changes raise the possibility for eventual effective and simple therapeutic application of Netrin-1.

True laminins are heterotrimeric structures named after the specific α , β and γ chains they use. Recent findings show that extracellular matrix components such as Lam511, Lam521, support the iPSC generation process and PSC self-renewal and increase iPSC generation efficacy (Lu et al, 2014a; Rodin et al, 2014; Rodin et al, 2010). Given that laminins, such as Lam511, were shown to be sufficient for mESC culture without any additional external stimulus (Domogatskaya et al, 2008), it will be interesting to determine if Netrin-1 can substitute for Laminin, or whether it would complement Laminin. Although the major laminin receptor on mouse ESC was described more than 25 years ago as an isoform of $\alpha 6 \beta 1$ (Cooper et al, 1991), it remains unclear whether it is sufficient to mediate the effects of laminin, since early studies focus principally on short term mechanical attachment, and the many domains of laminin suggest it has a number of signaling roles. Moreover, it is clear that laminins do not act alone, but collaborate with other cell surface receptor systems in a context dependent manner. For example, E-cadherin mediated cell contacts are also critical for iPSC generation and for pluripotency maintenance (del Valle et al, 2013; Koopmansch et al, 2013; Soncin et al, 2011).

The '2i' ground state inhibitors (MEKi and GSKi) are widely used in stem cell culture and blastocyst derivation. Interestingly, ICM cells derived from early blastocysts do not require MEK inhibitor if provided with this specific laminin substrate. This data affirms a critical guiding role for ECM proteins in early development. This finding suggests a more physiological alternative to ground state culture conditions. Moreover, the data suggests that formation of epiblast coincides with ERK-independent self-renewal *in-vitro* and consequent propagation as mESC lines (Boroviak et al, 2014). The results appear more significant since the use of prolonged ERK inhibition was recently shown to cause genomic instability and

impair the developmental potential of PSCs (Choi et al, 2017a). It would certainly be more physiological if one was able to replace small inhibitors that could have pleiotropic side effects with native extracellular matrix components. It will be important to determine whether this is a viable approach in the pursuit of high-quality iPSC generation, and even for daily PSC culture. To date, Lam 511 and Lam521 with the integrin $\alpha6\beta1$ receptor complex (CD49f/CD29) have been the most examined and have played key roles in maintaining PSC self-renewal. The data presented here makes a strong case that Netrin-1 may work at least equally to laminin.

While laminin is a heterotrimeric extracellular matrix protein of almost 900kDa, the laminin-like molecule Netrin-1 is much smaller, at 67kDa. As a secreted factor that acts in guidance, but can also interact with the ECM, Netrin-1 has the properties of other known matricellular proteins. These proteins are secreted into the extracellular environment, or matrix and modulate cell function by interacting with cell-surface receptors, proteases, hormones, and other effector molecules, as well as with structural matrix proteins such as collagens. The term 'matricellular' was introduced to explain the unusual diversity of functions that were beginning to be recognized in proteins such as thrombospondin-1 (TSP-1), SPARC, and tenascin-C. Furthermore, these proteins are highly expressed during development (Bornstein, 2009). Netrin-1, like laminin also signals through integrins, though it also binds a number of other receptors. It would be interesting to test whether integrins themselves could modulate iPSC generation and pluripotency regulation, alone or in combination with the other Netrin-1 receptors.

Mechanistically, Netrin-1 expression reduces p-ERK levels in mESCs. This finding further supports a role for Netrin-1 that is similar to Lam511 because Lam511 also allows ERK-independent self-renewal. However, Netrin-1 enhances Wnt signaling and laminin is not known to do this. As the promotion of Wnt signaling activation and ERK inhibition are the two key requirements for ground state conditions, I

would suggest that, Netrin-1 utilization as a matrix could inexpensively and positively regulate PSC self-renewal and mESC lines derivation.

We did perform co-culture experiments exploring the use of Netrin-1-expressing feeders, as well as with recombinant Netrin-1-coated surfaces to test mESCs self-renewal ability on these surfaces. These studies were not included in the manuscripts published as part of this thesis. These experiments were unsuccessful. This could be due to a number of possible reasons. For example, Netrin-1 may act in an autocrine manner in PSCs, with pairing occurring just after synthesis or secretion. It is also possible that recombinant Netrin-1, much like WNT, may not be stable as a molecule, or may be subject to post-translational modification. It would also be of interest to test mESCs derivation efficacy on Netrin-1 coated surfaces. These studies could be performed with and without ERK inhibitor and/or GSK inhibitor. There are several considerations if one were to take this type of approach. The concentration of Netrin-1 would need to be optimized, as a primary effect. Its interaction with existing stimuli would need to be characterized, and the impact on the resultant PSC properties would need to be evaluated. As an extension of these processes, its combination with other matrices might be explored.

How does the modulation of Netrin-1 signaling reconfigure naïve mESCs?

Netrin-1 signals via NEO1 and UNC5B receptors in pluripotent stem cells (PSCs). Among naïve PSCs, Netrin-1 enhances self-renewal, delays differentiation, increases WNT sensitivity and decreases responsiveness to FGF by triggering UNC5B internalization and subsequent degradation. Once a cell enters a state of intermediate or primed pluripotency, UNC5B is strongly elevated, and Netrin-1 stimulates lineage priming by potentiating FGF signaling. I will discuss the unresolved questions to try to answer how Netrin-1 confers to cells these particular configurations in different pluripotency states, and suggest further experiments and some hypothetical models.

What is the impact of Netrin-1 on lineage commitment?

The depletion of Netrin-1 negatively affects clonogenicity of mESC (ability to self-renew at clonal density), which suggest a role in differentiation. Accordingly, Netrin-1-depleted cells exhibit reduced differentiation and proliferation in the teratoma formation assay. Unfortunately, histological analyses of teratomas are not sufficiently quantitative to distinguish the deficiency of differentiation with respect to a defined lineage. Netrin-1 depletion inhibits differentiation into all 3 lineages in EBs assay. However, this assay does not test the differentiation capacity versus a defined lineage. It would be interesting to test the impact of Netrin-1 deletion into each specific defined lineage. These protocols could test a direct differentiation of the mESCs to ectoderm, endoderm and mesoderm. Assays to evaluate this are available, and could include cell sorting of reporter cell lines such as Rex1 (allowing tracking the exit from pluripotency) and optimized cell culture conditions using a different set of cytokines and inhibitors for each lineage (Mulas et al, 2017). It would be interesting to test these protocols to define more precisely the impact of Netrin-1 depletion for a defined lineage.

How is UNC5B receptor trafficking regulated in Netrin-1 expressing cells?

We identified that both Netrin-1 expression and ground state cell culture conditions, induced a strong decrease in UNC5B protein levels, but not in mRNA levels. This result strongly suggested a posttranslational control of receptor levels. As mentioned previously, Netrin-1-expressing cells cannot be differentiated to any lineages in teratomas. The netrin-1 expressing cells fail to differentiate properly for the mesoderm and endoderm and ectoderm lineages, while retaining pluripotency genes expression in EBs. Moreover, the cells are not responsive to FGF stimulation, which is the main cue guiding commitment. However, this impaired differentiation in EBs and the unresponsiveness to FGF is restored in cells that express a mutant

form of Netrin-1, which fails to interact with UNC5B. The resulting Netrin-1 and UNC5B expressing cells rescued the deficiency in FGF responsiveness, but failed to rescue EB formation. The mechanism underlying the requirement for UNC5B interaction was revealed in the extended studies in the thesis.

In particular, I found that Netrin-1 expressing cells induce the dynamin-dependent endocytosis and subsequent lysosomal degradation of the UNC5B receptor. In the absence of this signal, FGF-mediated signaling is restored, suggesting a functional link between UNC5B and FGF that was previously unsuspected as a regulator of pluripotency. It would be useful to perform Total Internal Reflection Fluorescence (TIRF) microscopy to directly visualize the dynamics of components of endocytic machinery, in real time in living cells, at the level of individual events that mediate this process. This approach is essential to elucidate the subtle changes that could modulate UNC5B endocytosis.

The endocytosis of the UNC5A, another member of UNC5 family, is mediated by Protein Kinase C α (PKC α) signaling in developing neurons (Bartoe et al, 2006). It would be interesting to perform a protein-based-screen to identify regulators of UNC5B endocytosis in response to increased Netrin-1 expression in naïve mESCs. This could help to modulate deregulated UNC5B protein expression levels, which seems to be required for robust lineage priming and which is altered in some pathology such as cancer (Liu et al, 2014).

These studies clearly identified a role for receptor trafficking and degradation in stem cell biology. Receptor trafficking is a regulatory process in several physiological and pathological contexts. However, receptor trafficking in embryonic stem cells and resulting cell fates is essentially undiscovered. Our work suggests that dynamic receptor trafficking exists in mESCs and this can affect resulting cell fates. Such a broad biological mechanism seems unlikely to be specific to Netrin-1 signaling, and probably plays a role for the regulation of other signaling pathways.

Could dynamic trafficking take place for other receptors regulating distinct pluripotency stages?

When we analyzed the available RNA-sequencing data in mESCs, we observed that receptor trafficking could be a mechanism in the control of pluripotency. FGFR1 mRNA expression levels are very high in 3.5 dpc ICM, 4.5 dpc, 5.5 dpc epiblast, 2i-induced mESCs and in growth arrested (diapause) epiblast (Data analysis of RNA-sequencing (Boroviak et al, 2015)). However, FGF/ERK signaling mediates inhibitory effects on naïve pluripotency maintenance and self-renewal on mESCs. Furthermore, as FGF/ERK signaling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment (Kunath et al, 2007), a paradox is revealed. Why the naïve pluripotent embryos and mESCs do not down-regulate FGFR1 gene remains unknown. It seems the clearest and simplest mechanism to control FGF signaling, and suggests that keeping FGFR present and the cells in a potentially receptive state is important.

On the other hand, it has been established that FGFR1 protein levels can be controlled by endocytosis. Ligand (FGF) binding to FGFR1 (along with heparin/HSPG) induces receptor dimerization, activation of its kinase activity and autophosphorylation of multiple cytoplasmic tyrosine residues to further activate intracellular signalling, including the PI3K/AKT and Ras/ERK pathways (Lemmon & Schlessinger, 2010). Additionally, FGFR1 phosphorylation initiates its removal from the plasma membrane and promotes its subsequent lysosomal degradation, resulting effectively in receptor downregulation (Sorokin et al, 1994). A number of candidate proteins may play roles in this regulation. Nedd4-1 (neuronal precursor cell developmentally downregulated 4-1), a member of E3 ubiquitin family, binds and ubiquitylates the activated FGFR1 to control its endocytosis and function (Sorokin et al, 1994). Interestingly, the pluripotent 4.5 dpc, 5.5 dpc epiblast, 2i-induced mESCs and in growth arrested (diapause) epiblast exhibit very high levels of Nedd4 expression. If Nedd4 does regulate FGF4 in stem cells, it would be the first report that FGFR1 trafficking regulated by FGF4 and Nedd4 regulates stem cell fates.

These observations, together with the observation of high levels of expression of several E3 ligases in mESC and early epiblast suggest receptor trafficking could be a major regulator of pluripotency and commitment. These hypotheses provide fertile ground for future investigators.

How does UNC5B endocytosis occur in PSCs? Could expression of a single ligand internalize a receptor, or are receptor(s)-ligand(s) complex formed?

While not shown in this thesis, among the Netrin-1 expressing mESCs cells, the most affected pathway according to KEGG analysis was the lysosomal pathway (data not shown). This finding was not a focus of the current study, but looking backwards it is perhaps not completely unexpected. In fact, it was known that Netrin-1-mediated chemoattractive and chemorepulsive responses during neuronal development involve endocytosis-mediated receptor trafficking within the growth cones. Moreover, it was suggested that a tight control of endocytosis may be a necessary aspect of guidance receptor activation and signaling.

Sustained Netrin-1 expression in mESC does not allow lineage factor expression. An increase in UNC5B at the transcript level alters differentiation ability of mESCs. Therefore, I hypothesized that altered UNC5B surface protein levels, and overall UNC5B expression, could act to block the exit from pluripotency and lineage commitment in Netrin-1 expressing mESCs. Consistent with this model, even hypomorphic alleles of UNC5B are lethal. To test this, Netrin-1 and inducible-UNC5B expressing mESCs were established. These cells rescued the deficiency in FGF responsiveness of Netrin-1 mESCs. However, in our studies this was not sufficient to rescue the impaired differentiation completely. This may be due to a possible lack of stability of UNC5B induction.

In the simplest model; UNC5B may be a direct mediator of p-ERK activity in mESCs (**Figure19-Model-I**). To investigate whether p-ERK is a downstream target of

UNC5B, we used an shRNA approach to delete UNC5B. However, depletion of UNC5B did not affect the p-ERK levels in mESC, indicating that UNC5B is not a direct regulator of p-ERK levels in mESCs. This result suggests a more complex model than the single receptor model. A second possibility based on known roles in neuronal development suggests that, upon sustained Netrin-1 expression, UNC5B is not the sole Netrin-1 receptor that is internalized and thus the trafficking of other receptors might also be influenced. For example, UNC5B and FGFRs (in this case FGFR1, as it is the sole receptor expressed in mESCs) could be associated together in the membrane and then would be internalized as a complex. The internalization of receptor complexes has already been shown for shown EphB2 and ephrinB1 after binding their ligands (Zimmer et al, 2003).

Which receptors may form complexes with UNC5B in mESCs to regulate p-ERK levels?

Notably, several studies show an association between Unc5 receptors and FLRT receptors, which directly modulates FGF/ERK signaling. FLRTs are broadly expressed during vertebrate development and adulthood and were first identified as modulators FGFR signaling (Bottcher et al, 2004; Haines et al, 2006). The FLRT1-3 proteins recently emerged as cell adhesion molecules (CAMs) that also interact with repulsive cell guidance receptors of the Unc5 family (Karaulanov et al, 2009; Lu et al, 2015; Sollner & Wright, 2009; Yamagishi et al, 2011). FLRTs are transmembrane receptors, whose extracellular region contains an N-terminal Leucine-Rich-Repeat (LRR) domain, a linker region, a FN domain and a juxtamembrane linker (Yamagishi et al, 2011). The LRR domain of FLRT, mediates FLRT-FLRT and FLRT-Unc5 interactions. A crystallography study, revealed the structure of interaction domains between FLRT and Unc5 receptors. The FLRT2 LRR form complexes via Unc5d Ig1 domain and FLRT3 LRR form complexes with UNC5B Ig1 domain (Seiradake et al, 2014). Moreover, the FLRT3 receptor interacts with FGFR1. This is notable that FGFR1 is the sole FGFR present on mESC (Bottcher et al, 2004). In the light of these findings, we can suggest a model in which a complex

composed of UNC5B-FLRT3-FGFR1 modulates FGF response in mESCs. In this case, upon Netrin-1 binding to UNC5B, a complex composed of UNC5B-FLRT3-FGFR1, that harbors pro-commitment activities, subsequently becomes internalized and degraded. **(Figure-19 Model-II)** .If this second model is the effective model, one role of elevated levels of Netrin-1 is to promote the degradation of these receptors complexes in mESCs, thus preventing FGF signaling indirectly and maintaining pluripotency. In agreement with this model, sustained Netrin-1 expression does not allow effective exit from pluripotency. In this case, the pluripotency genes (Sox2, Nanog, Esrrb) continue to be expressed, while the UNC5B promoter is still repressed during mESCs differentiation. It will be important to demonstrate how Netrin-1 affects receptor trafficking to promote stemness, as this mechanism may also be conserved in pathological context such as cancer. For this reason, interventions targeting Netrin-1 interaction with UNC5B could offer a way to treat cancer cells and promotes differentiation and/or apoptotic responses (Grandin et al, 2016b). In particular, the tumor-initiating cells (so called cancer stem-like cells) have been associated with tumor resistance to classic chemotherapies. The failure of these therapies is the principal cause of death in cancer. Targeting this problematic cell population could be of significant use for clinical applications.

According to the classical view, dependence receptors induce apoptosis when they are unbound by Netrin-1. In the case of cancers that exhibit higher levels of Netrin-1 expression and lower receptor activity, targeting Netrin-1/UNC5B interaction is not expected to trigger UNC5B-induced apoptosis. Our results indicate that blocking the Netrin/UNC5B interaction could affect receptor trafficking leading to accumulation of UNC5B on the cell surface and triggers apoptosis and/or differentiation. To date, dependence receptors are shown to induce actively apoptosis. However, it is also worth considering, in response to reduced ligand expression levels, if they can mediate differentiation of cancer cells that possess similar characteristic to cancer stem cells, or whether they would universally promote cancer cell survival.

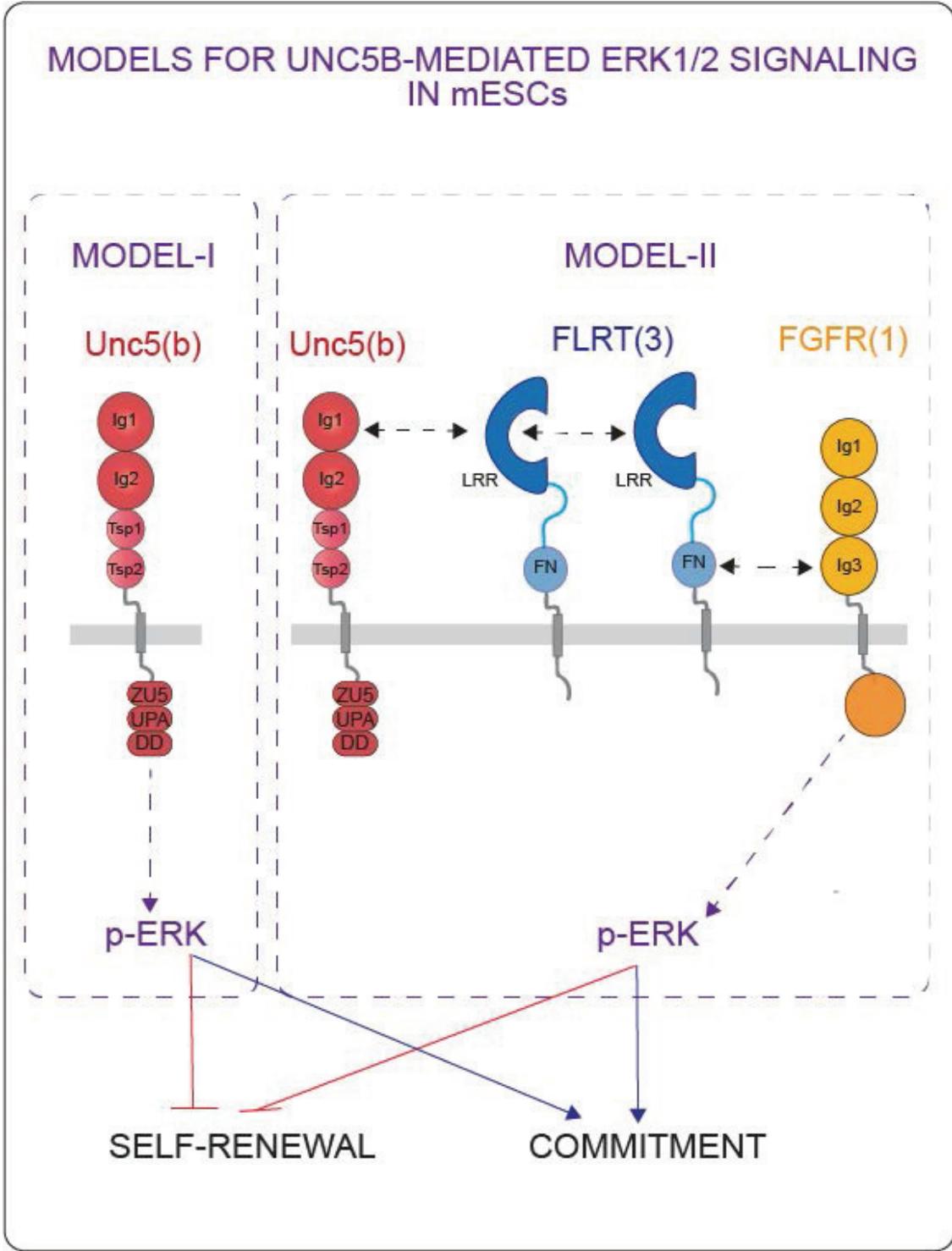


Figure-19: Models for UNC5B-mediated ERK1/2 signaling in mESCs.

How co-regulations between Netrin-1 signaling and WNT signaling takes place?

Our work showed that Netrin-1 expression is tightly regulated by WNT pathway factors. In mESCs, these include the repressor Tcf7l1 and the activator Lef1 in mESCs. Perhaps more importantly, Netrin-1 expression promotes Wnt signaling activation and increases Wnt sensitivity in mESCs. These results provide the initial description of the interactions between these two pathways in mammalian cells. However, the expression of NETRIN and WNT described in the literature generally coincides, which strengthens our view about the collaboration between these two signaling pathways. Both Netrins and WNT proteins are expressed early in developmental stages. Both promote mESC self-renewal. And interestingly, their expressions are not restricted to the PSCs as both are also expressed in several adult tissues. Netrins and WNT proteins co-regulate axon guidance, cell migration and inner ear development (Killeen & Sybingco, 2008; Levy-Strumpf, 2016; Riccomagno et al, 2005; Salminen et al, 2000).

Both WNT proteins and Netrins can play key roles in tumorigenesis. Wnt signals are crucial for the activity of the epithelial stem cells and Wnt pathway mutations are frequently observed in carcinomas. The APC gene, a key downstream regulator of WNT, was identified as being mutated in a hereditary colon cancer syndrome termed familial adenomatous polyposis (Kinzler et al, 1991). Several sporadic colorectal cancer result from loss of both APC alleles (Kinzler & Vogelstein, 1996). Loss of APC function leads to the inappropriate stabilization of β -catenin and results with the formation of constitutive complexes between β -catenin and Tcf factors (Korinek et al, 1997). Similarly, mutations in Axin2 gene cause predisposition for colorectal cancers (Lammi et al, 2004). Mutations in β -catenin gene are also reported in colon cancer (Morin et al, 1997) and melanoma (Rubinfeld et al, 1997). Netrin-1 signaling is also altered in lung, neuroblastoma, breast, colon and several other cancer types (Goldschneider & Mehlen, 2010). Interestingly, The DCC mice carrying the D1290N that is crossed with the APC^{+ / 1638N} mutant background predisposed to form adenocarcinomas but not metastases, exhibited the serosal

invasion and micrometastases due to the loss of DCC apoptotic activity (Castets et al, 2011). It would be interesting to further investigate if Netrin-1 exhibits increased expression in the APC^{+ / 1638N} mutant mice and potentiated the effect of D1290N DCC mutation.

There are other clues that validate the pathways revealed in my thesis. For example, Wnt-Frizzled mediates down-regulation of Unc5 'signaling' in *C.elegans*. This observation might now more simply be explained instead via the induction of netrin and the subsequent down-regulation of Unc5. Supporting this notion, the signaling is rescued in the worm, as in our mESC system, by forced expression of UNC5B. Thus, the general mechanism shown here is not likely to be unique to mESC, but a general biological process that stem cells use to regulate pluripotency.

Summary

Together, these studies served to reveal the novel role of Netrin-1 in embryonic stem cells. The regulation of its function at the receptor level has important implications for future study across this field. Biologically, the results provide insight into other systems, all the way back to *C.elegans*. Finally, the results further suggest additional practical options for day-to-day ESC culture and provide new areas to explore. To better understand Netrin-1 signaling in stem cells, it would be very useful to perform a protein-based screen to identify the potential Netrin-1 pathway interactors. Approaches such as TAP (Tandem Affinity Purification) method combined with mass spectrometry could be suitable to identify components of these protein complexes. The potential for new understanding is great. It would be a good time to be a new PhD student starting again.

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