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# MASTER THESIS

## Exploring the role of Groucho-related gene 5 (Grg5) in self-renewal and neuronal differentiation of embryonic stem cells

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# Contents

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# Abstract

ESCs are characterized by two important properties: self-renewal and pluripotency. Deciphering the molecular mechanisms that regulate these two processes is a continuing inquiry appealing the interest of stem cell research. The identification of new factors as essential regulators contributes to the interpretation of ESC biology. Among other pathways, specific interest has been focused on the underlying mechanisms that govern ESC neural differentiation since their unraveling can permit the study of CNS development *in vitro* and help regenerative neurogenesis.

Groucho Related Gene 5 (GRG5) is a truncated member of the murine Groucho family. GRG5 was initially characterized as antagonist of the co-repressive activity of the other family members (long GRGs), hence acting as a "de-repressor". However, later studies have shown that it is a multifunctional protein with important role in diverse cellular processes and postnatal developmental procedures. *Grg5* has been reported as a direct target of STAT3 in ESCs; however its role has not been elucidated yet.

This study unveils GRG5 function as a critical component of the regulatory network of ESC pluripotency and its involvement in ESC differentiation. Loss and gain of function approaches demonstrate that GRG5 acts as a self-renewal promoting factor in ESCs by positively regulating the expression of pluripotency factors. Consequently, depletion of GRG5 deregulates the ESCs pluripotent state whereas its forced expression enhances ESC self-renewal. Interestingly, OE GRG5 ESCs exhibit cancer cell-like properties, implying also the implication of GRG5 in tumorigenicity.

Moreover, we delineate GRG5 involvement especially in ESC neuroectodermal commitment via Wnt and BMP signaling suppression, pathways that are known to inhibit ESC neural specification. Specifically, we present for the first time the physical association of crucial mediators of these pathways with GRG5 unraveling the molecular mechanism of the observed phenomenon and thus tracing its executive role in neural fate determination.

# Περίληψη

Τα εμβρυϊκά βλαστοκύτταρα (ΕΒΚ) χαρακτηρίζονται από δύο σημαντικές ιδιότητες: την αυτοανανέωση και την πλειοδυναμία. Η αποκωδικοποίηση των μοριακών μηχανισμών που ρυθμίζουν αυτές τις δύο διαδικασίες είναι ένα διαρκές ερώτημα που συγκεντρώνει το ενδιαφέρον της έρευνας στον τομέα των βλαστοκυττάρων. Ο προσδιορισμός νέων ρυθμιστικών παραγόντων συμβάλλει στην καλύτερη κατανόηση της βιολογίας των ΕΒΚ. Μεταξύ άλλων μονοπατιών, πολλές μελέτες έχουν επικεντρωθεί στους υποκείμενους μηχανισμούς που διέπουν τη νευρική διαφοροποίηση των ΕΒΚ, δεδομένου ότι η αποκρυπτογράφησή τους μπορεί να συμβάλλει στη μελέτη της ανάπτυξης του Κεντρικού Νευρικού Συστήματος *in vitro* καθώς και στην ανάπτυξη νέων μεθόδων αναγεννητικής νευρογένεσης.

Η πρωτεΐνη Groucho Related Gene 5 (GRG5) ανήκει στα «ημιτελή» μέλη της οικογένειας Groucho στο ποντίκι. Η πρωτεΐνη GRG5 αρχικά χαρακτηρίστηκε σαν ανταγωνιστής της συν-κατασταλτικής δράσης των υπόλοιπων μελών της οικογένειας, δρώντας ως εκ τούτου σαν «από-καταστολέας». Ωστόσο, μετέπειτα μελέτες έδειξαν ότι η πρωτεΐνη GRG5 είναι μια πολύ-λειτουργική πρωτεΐνη η οποία έχει ενεργό ρόλο σε διάφορες κυτταρικές και αναπτυξιακές διαδικασίες. Στα ΕΒΚ έχει φανεί ότι η το γονίδιο Grg5 είναι άμεσος στόχος του μεταγραφικού παράγοντα STAT3 και ρυθμίζεται θετικά, ωστόσο η λειτουργία της GRG5 δεν έχει μελετηθεί περαιτέρω στο σύστημα αυτό.

Στην παρούσα μελέτη δείχθηκε ότι η GRG5 λειτουργεί σαν σημαντικός παράγοντας στο ρυθμιστικό δίκτυο που διαμορφώνει την πλειοδύναμη κατάσταση των ΕΒΚ καθώς και στην διαφοροποίηση τους. Με πειράματα υπο-/υπερ-έκφρασης της GRG5 αποδείχθηκε ότι η GRG5 συμβάλει στην αυτοανανέωση των ΕΒΚ μέσω θετικής ρύθμισης της έκφρασης παραγόντων πλειοδυναμίας. Συνεπώς, η εξάλειψη της GRG5 αποσταθεροποιεί την κατάσταση πλειοδυναμίας των ΕΒΚ ενώ αντιθέτως η υπερεκφρασή της οδηγεί στην αυξημένη αυτοανανέωσή τους. Ενδιαφέρον παρουσιάζει ότι τα ΕΒΚ που υπερεκφράζουν την GRG5 παρουσιάζουν καρκινικές ιδιότητες, καταδεικνύοντας τον ρόλο της GRG5 στην ογκογένεση.

Επιπλέον, περιγράφηκε ο σημαντικός ρόλος της GRG5 στην νευρική διαφοροποίηση των ΕΒΚ μέσω καταστολής των μονοπατιών Wnt και Bmp, τα οποία είναι γνωστό ότι παρεμποδίζουν τη επαγωγή νευροεκτοδέρματος. Συγκεκριμένα, παρουσιάζουμε για πρώτη φορά την φυσική αλληλεπίδραση σημαντικών παραγόντων αυτών των μονοπατιών με την GRG5, συμβάλλοντας έτσι στο «ξεδίπλωμα» του μοριακού μηχανικού πίσω από το παρατηρούμενο φαινόμενο με σκοπό την κατάδειξη του σημαντικού ρόλου της στον καθορισμό της νευρικής κυτταρικής τύχης.

# Introduction

## 1. Embryonic stem cells

The development of multi-cellular organisms begins from a single-celled zygote. The zygote undergoes a process called cleavage, which is marked when the zygote divides through mitosis into two cells, and is characterized by multiple rapid cell divisions. Initially the dividing cells, called blastomeres, are undifferentiated and aggregated into a sphere enclosed within the membrane of glycoproteins (termed the zona pellucida) of the ovum. At the stage of the eight cells (early morula), blastomeres are subjected into symmetrical cleavage divisions and start to bind firmly together in a process called compaction until the stage of 32 cells (morula). Cells develop tight gap junctions and become polarized in a radial direction, enabling them to develop in an integrated way and co-ordinate their response to physiological signals and environmental cues. During this period, a permeability seal between the inside and outside of the embryo, and a fluid-filled cavity, the blastocoel begins to form in the interior of the embryo. The blastocoel expands and the embryo becomes a blastocyst, which consists of an outer cell layer of epithelial morphology called the trophectoderm (TE) and a clump of cells in the interior, the inner cell mass (ICM). Up to the time of blastocyst formation, the fate of individual cells can be altered by changing their position within the embryo, indicating a flexible cell fate potential of blastomeres [1]. At the stage of late blastocyst, ICM cells that are exposed to the fluid of the blastocoel develop into hypoblast (Primitive endoderm, PE), while the remaining cells become the epiblast (EPI). Throughout the course of development, the epiblast gives rise to the future fetus, whereas the TE and PE form extraembryonic tissues including placenta and yolk sack, respectively.

Development begins after fertilization which results in a single totipotent cell. This zygote divides into identical totipotent blastomeres, which can later develop into any of the three germ layers of an embryo (endoderm, mesoderm, or ectoderm), or into cells of the placenta. After reaching a 16-cell stage, the totipotent cells of the morula differentiate into cells that will eventually become either the blastocyst's ICM or the outer trophoblasts. Approximately four days after fertilization and after several

cycles of cell division, these totipotent cells begin to specialize. The ICM, the source of embryonic stem cells, becomes pluripotent and do not give rise to extraembryonic tissues.

Pluripotent mouse embryonic stem cells (ESCs) were firstly isolated in 1981 [2, 3]. ESCs are derived from the ICM of the pre-implantation blastocyst (E3.5) and in specific culture conditions they can be maintained indefinitely in an undifferentiated state. ESCs have two characteristic features, pluripotency and self-renewal. Although ES cells are clearly not identical to ICM cells, they maintain the ability to give rise to all three germ layers, and when injected into blastocysts they behave in the same manner as the host ICM cells and participate in embryonic development, providing a universal cell source for the study of cell biology of embryogenesis. Moreover, ESCs are able to form teratomas consisting of well-differentiated tissues or embryonic less-differentiated tissues, when they are injected into immunocompromised individuals [4]. Importantly, they can be incorporated to the ICM of the pre-implantation embryo resulting in chimeric animals [5].

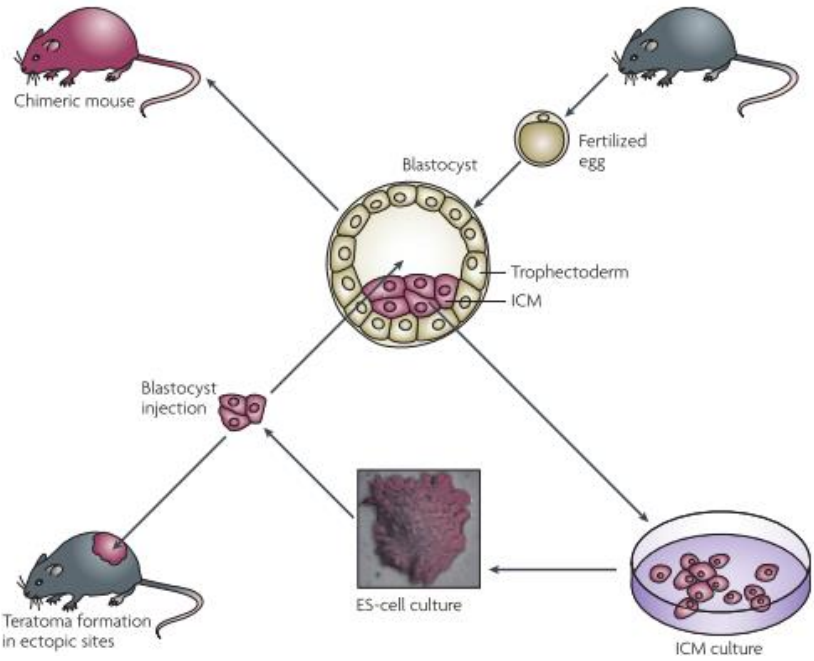


**Figure 1: Embryonic stem cell derivation and features.** ESCs are derived from the ICM of the pre-implantation murine blastocyst and can be cultured *in vitro* under appropriate conditions. They are able give rise to the three germ layers after injection into blastocyst, generating chimeric mice. ESCs have also the ability to give rise to teratomas upon injection to immunocompromised mice [5].

A distinct pluripotent cell population can be isolated from the post-implantation epiblast (E6.5), termed EpiSCs [6, 7]. EpiSCs have different transcriptomic profile, pluripotency markers, clonogenicity potential and growth factor requirements in comparison to ESCs. Also, they can generate teratocarcinomas but do not contribute effectively to blastocyst chimeras [8]. All the above considered, there are two discrete states of pluripotency throughout embryonic development, “naïve” or “ground” and “primed”. Mouse ESCs comprise the “ground” state of pluripotency. Conventional generation of stem cells from human blastocysts produces a developmentally advanced stage of pluripotency, resembling the features of mouse EpiSCs, thus mouse EpiSCs and human ESCs represent the “primed” state of pluripotency. However, it has been reported recently that the isolation of human naive embryonic stem cells directly from the human ICM is feasible and also their propagation under appropriate culture conditions (inhibitors of MAPK/Erk, GSK3, and PKC), maintaining the naïve characteristics [9]. In addition, a very promising approach of direct conversion of human pluripotent stem cells (PSCs) to a more naïve developmental status via transient histone deacetylase inhibition has been reported by the same group, offering a more practical experimental system for investigation of human pluripotency progression [10].

## 2. ESC pluripotency regulation

ESC pluripotency state is controlled in multiple levels including mainly transcription factors, signaling pathways and epigenetic mediators. Together with developmental factors, these should function dynamically and coordinately in order to maintain pluripotency or enable rapid differentiation upon appropriate stimulation.

### 2.1 Transcriptional regulation

Several transcription factors have been identified to be essential for ESC pluripotency. In ESCs, the pluripotent state is mainly regulated by the core transcription factor trio of Oct4, Sox2 and Nanog [11, 12, 13]. These transcription factors function together in two ways in control of ESC state. Firstly, they positively regulate

their own promoters, forming an interconnected autoregulatory loop and secondly they co-occupy and activate expression of genes necessary to maintain ESC state, while contributing to repression of genes encoding lineage-specific transcription factors whose absence helps prevent exit from the pluripotent state [14]. Also, these so-called master regulators associate with signaling pathways mediators and chromatin remodeling proteins to form a complex network that maintains ESC undifferentiated state [15].

**OCT4**

Oct4 is encoded by the Pou5f1 gene and it belongs to the Pit-Oct-Unc (POU) family of homeodomain proteins. It is exclusively expressed within the totipotent mouse blastomeres, pluripotent epiblast as well as primodial germ cells (PGCs) [16, 17]. Oct4 null embryos fail to form a pluripotent ICM and do not develop beyond the blastocyst stage, but rather, differentiate into trophectodermal tissue [13]. Thus, Oct4 plays a critical role in the establishment and maintenance of pluripotency. Oct4 binds to the octameric DNA sequence ATGCAAAT often together with its transcription partner SOX2, which binds to a neighboring sox element, to induce the expression of pluripotency related genes [18] and repress the expression of developmental genes [14]. Interestingly, Oct4 overexpression leads to differentiation into primitive endoderm and mesoderm [19] whereas sustained upregulation of Oct3/4 in ES cells in serum-free LIF-deficient medium causes efficient neuroectoderm formation and subsequent neuronal differentiation, suggesting that Oct4 transcriptionally regulates genes involved in coordinating multiple cellular functions [20].

**SOX2**

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encodes transcription factors with a single HMG DNA-binding domain. SOX2 belongs to the SOX B1 subgroup, which also includes SOX1 and SOX3, based on homology within and outside the HMG box [21]. Initially, SOX2 expression is detected at morula stages and later it is found within the ICM of the blastocyst. Expression persists throughout the epiblast and in a band of extraembryonic ectoderm reaching the

embryonic/extraembryonic boundary [22]. Upon neural plate formation, SOX2 expression is restricted to a region of anterior ectoderm which will give rise to neuroectoderm and anterior surface ectoderm in later stage. In the developing embryo, Sox2 RNA is detected throughout the brain and neural tube among others indicating an additional role in neurodevelopment. Its essential role in development is also apparent since Sox2 null embryos die immediately after implantation due to impaired epiblast formation [23].

Regarding the role of SOX2 in ESC pluripotency, SOX2 is critical for maintaining the pluripotent state since reduction of SOX2 expression is associated with a loss of pluripotency and a propensity for differentiation. Specifically, Sox2-null embryos fail to give rise to ES cells, instead they differentiate primarily into trophectoderm [23]. The function of SOX2 in regulating the expression of most pluripotency-associated genes depends on the OCT3/4 synergistic activation of an enhancer containing Oct3/4 and Sox2 binding motifs. However, SOX2 was dispensable for the activation of this kind of enhancers since they remained active even after SOX2 depletion, indicating a functional redundancy of Sox factors on these Oct–Sox enhancers [24]. Importantly, SOX2 has a crucial role in regulating neural induction, maintaining the self-renewal and differentiation of neural progenitor cells, by repressing key regulators of other lineage fates, such as brachyury (T) [25].

**NANOG**

NANOG is a unique homeodomain transcription factor first identified and characterized in mammalian pluripotent cells and developing germ cells [11, 12]. Nanog shares homebox similarity with those of the Nk-2 gene family, however sharing less than 50% amino acid identity, indicating that NANOG is completely distinct from the NK-2 family [12].

After functional screening, NANOG was considered as the third member of the core pluripotency network since it enables LIF-independent ESC self-renewal maintenance. Nanog mRNA is initially identified in the stage of compacted morula and afterwards in the ICM of the blastocyst (ESCs) [11]. In the ICM stage, antagonistic expression between Nanog and Gata6 leads to the determination of primitive endoderm versus pluripotent epiblast cell fate, restricting NANOG expression in the

latter [12]. NANOG seems to have an active role mainly in pluripotency maintenance rather in pluripotency establishment, since NANOG-null embryos are able to generate pluripotent cells. However, these cells can not maintain their undifferentiated character thus spontaneously differentiate into extra-embryonic endoderm [12]. Further studies showed that NANOG expression levels acts as a rheostat regarding the ESCs differentiation options, regulating the self-renewal efficiency [26].

As we stated above, OCT4, SOX2, and NANOG are thought to be the transcriptional master regulators that specifies ES cell identity because of their unique expression patterns and their indispensable roles during early development. Nevertheless, after years of research in ESC biology, the transcriptional network which regulates self-renewal and maintenance has been further investigated. KLF4, NR0B1 and c-MYC are some critical members of this network, cooperatively functioning with the core pluripotency factors.

KLF4, together with other members of Klf-family, is highly expressed in ESC and is downregulated in EpiSC, indicating that Klf-family members may not have a critical role in regulating pluripotency rather they are important for the self-renewing state of ESC. Klf protein circuitry integrates with Nanog circuitry by co-occupying many common binding loci of ES-cell specific genes thus exhibiting a highly interconnected transcriptional regulatory network [27]. Notably, overexpression of KLF4 in EpiSC under ground state conditions, resulted in re-expression of endogenous Klf4 and other ES-cell specific transcripts and it was sufficient for stable reprogramming of EpiSC to naïve ESC [28].

NR0B1, or DAX1, has also implicated in the transcriptional network controlling ESC pluripotency. NR0B1 acts predominately as a transcriptional repressor in mouse ESC as Nr0b1 knock-down resulted in multilineage differentiation of ES cells, indicating that Nr0b1 plays an important role in their self-renewal [29]. NR0B1 interacts with OCT3/4 through its DNA-binding domain thus negatively regulating its transcriptional activity. Hence, NR0B1 acts as a “fine tuner” of OCT3/4 activity, maintaining its expression in an appropriate level in ES cells [30]. In addition, Nr0b1 is required for the

final stage of somatic cell reprogramming, aiding in the acquirement of induced pluripotency [31].

C-MYC has been also proposed as a key controller of regulatory processes such as maintenance of pluripotency and lineage specification. MYC belongs to a family of helix-loop-helix/leucine zipper transcription factors and through heterodimerization with its binding partner MAX, normally regulates biological processes like cell proliferation, transformation, growth, differentiation and apoptosis [32]. During early embryonic development, C-myc is essential since C-myc-null embryos die by E10.5, displaying defects in the developing heart, neural tube, vascular and hematopoietic systems. Also, C-myc is required for ESC self-renewal and maintenance possibly by functioning as a downstream target of LIF/STAT3 pathway. A proposed mechanism is that C-MYC integrates signals transduced by LIF and Wnt and acts directly on target genes [32]. In addition, c-myc has important role in ESC primary differentiation since myc $^{-/-}$ ES cells fail to generate normal sized EBs [33]. Importantly, C-MYC is one of the factors along with OCT4, SOX2 and KLF4 which upon ectopic expression are sufficient for reprogramming mouse embryonic and adult fibroblasts to induced pluripotent cells (iPS) [34].

## 2.2 Signaling pathways

Signaling cascades start upon an extrinsic stimulus at the cell surface and terminate in the nucleus with the activation or repression of transcription thus allowing cells to respond to environmental cues. In the developing embryo, signaling pathways play a critical role for the cell positional establishment and differentiation. The main signaling pathways which are associated with ESC pluripotency and self-renewal are LIF/JAK/STAT3, Wnt/β-CATENIN and Bmp. They can also regulate ESC fate determination when differentiation is dictated. The combinatorial role of signaling pathways and transcription factors is the key for pluripotency maintenance and differentiation.

**LIF/JAK/STAT3 signaling**

Leukemia Inhibitory Factor (LIF) is one of the cytokines which comprise IL-6 cytokine family together with IL-6, IL-11, IL-27, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1 and NNT/BSF-3/CLC. Smith and colleagues demonstrated that mESCS is dependent on paracrine LIF signaling from feeder cells of mouse embryonic fibroblasts for their self-renewal [35]. Since then LIF is provided by a feeder layer of embryonic fibroblasts or as a recombinant protein for feeder-free culture conditions and is sufficient to maintain mESCs propagation. Upon binding to its receptor, LIF can trigger three pathways in mESCs: the JAK/STAT3, the PI3K/AKT and SHP2/MAPK pathway [36]. However, only JAK/STAT3 is essential for LIF-dependent self-renewal of ESC, since STAT3 activation was sufficient to maintain the undifferentiated state of ES cells in LIF-free conditions [37].

LIF signaling starts upon LIF binding to the low-affinity LIF receptor β (LIFRβ) which then heterodimerizes with the signal transducer glycoprotein 130 (gp130). This complex activates Janus-associated tyrosine kinases (JAKs) which phosphorylate tyrosine residues on the cytoplasmic domain of gp130. The phosphorylated tyrosines create docking sites for SH2 domain of STAT3 and the recruited STAT3 is phosphorylated by JAKs on Tyr705 resulting on its homodimerization [38, 39, 40]. The STAT3 dimer is translocated to the nucleus and bind to STAT3-binding sites in the promoter/enhancer of targets genes thus activating their transcription [41, 42].

Unraveling the functional role of STAT3 in mESCs, ChIP analysis revealed that STAT3 binds to the promoters of many pluripotency genes such as Oct4, Nanog, Klf4 and C-myc. In addition, one third of STAT3 binding sites are co-occupied by the three master regulators OCT4, SOX2 and NANOG, indicating that STAT3 is a crucial component of gene regulatory mechanism of pluripotency [42, 43]. STAT3 targets genes include developmentally regulated tissue-specific genes such as Gata3, Gata4, T (brachyury) and Eomes and their STAT3-mediated suppression contributed to the prevention of mESC differentiation into endoderm and mesoderm lineages [44, 45]. Importantly, JAK/STAT3 overactivation reinforces the conversion of EpiSCs into naïve pluripotent cells and diminishes the requirement of additional pluripotency culture conditions enabling the reprogramming of somatic cells [46].

**TGF-β/SMAD signaling**

The TGF-β superfamily of growth factors is divided into two subgroups based on the signaling cascade they can activate, the TGF-β/Activin/Nodal and BMP/GDF/MIS pathway [47]. The TGF-β family members are evolutionary conserved and exert a wide range of biological processes in various cell types. For instance, they can regulate cell proliferation, fate decision, differentiation and apoptosis and many of them function during embryonic development for pattern formation and tissue specification.

Both TGF-β signaling pathways are initiated upon TGF-β family members binding to the trans-membrane type I and type II serine/threonine receptors on the cell surface. Each member of the TGF-β superfamily binds to a unique combination of type I and type II receptors. Type I receptors are also termed activin receptor-like kinase (ALK) and ALK4, ALK5, ALK7 transduce canonical TGF-β signals, whereas ALK1, ALK2, ALK3 and ALK6 transduce BMP signals. Ligand first bind to the type II receptors with low affinity and type I receptors is recruited to form a heteromeric complex. Type II receptors phosphorylate type I receptors in its GS domain, stabilizing the complex. Then the serine/threonine kinase activity at the intracellular domain of the type I receptors phosphorylates the SMADs transcription factors which undergo homo-trimerization [48]. The latter complex forms a complex with the co-SMAD4 and translocate into the nucleus to control transcription of target genes. The Activin/Nodal branch leads to the activation of the SMADs 2/3 whereas the BMP activates the SMADs 1/5/8 [49].

BMP4 which activates BMP signaling has a crucial role in the maintenance of ES cell identity since LIF alone is insufficient to sustain self-renewal of mESCs [45]. BMP4 inhibits neural specification of mESCs by inducing the expression of negative helix-loop-helix inhibitor of differentiation (Id) family members. IDs can suppress neural differentiation by inhibiting pro-neural bHLH factors such as MyoD and Mash1 [45]. Therefore, LIF/STAT3 and BMP/SMAD signaling have combinatorial function to sustain ES cell self-renewal whereby IDs act in a lineage-specific manner, suppressing neural determination, complementing the STAT3-mediated blockage of mesoderm and endoderm commitment. Noteworthy, genome-wide occupancy analysis revealed that many of SMAD1 target genes overlap those of the core pluripotency

factors, OCT4, SOX2 and NANOG indicating that BMP signaling has a synergistic role in pluripotency regulation [50].

**Wnt/β-catenin signaling**

Wnt signaling contributes to the regulation of multiple cellular processes including proliferation, cell polarity and organogenesis. In addition, Wnt signaling has an essential role in maintenance of pluripotency and differentiation of embryonic stem cells as well as the self-renewal of adult stem cells in multiple tissues 51. Importantly, canonical Wnt signaling is required for primitive streak and anterior-posterior (AP) axis formation in early development. In addition, Wnt signaling has an essential role in mesoderm lineage specification and inhibits neuronal differentiation [52, 53, 54, 55, 56, 57].

In the absence of Wnt ligand, a complex consisting of AXIN, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3β), is formed. B-CATENIN binds to this destruction complex and it is constitutively and sequentially phosphorylated by CK1 and GSK3β. Upon phosphorylation, β-CATENIN becomes susceptible to ubiquitination and subsequent proteasome-mediated degradation [58]. On the contrary, when WNT ligands binds to the receptors Frizzled and LRP (low-density lipoprotein receptor protein) 5/6, GSK3β is inhibited and the assembly of the destruction complex is hindered, resulting in the stabilization of β-CATENIN. Therefore, β-catenin accumulates in the cytoplasm and translocates to the nucleus, interacts with transcription factors of the TCF/LEF family and activates downstream genes including Axin2, Cdx1, T, c-Myc and CyclinD1 [59].

Wnt signaling was first implicated in the regulation of pluripotency and self-renewal of mESCs after the demonstration that its activation through inhibition of GSK3β, by 6-bromoindirubin-3'-oxime (BIO), was able to maintain pluripotency in absence of LIF [60]. TCF3 (or TCF7L1) is the most expressed of the TCF/LEF factors in mESCs and is a negative regulator of ES cell pluripotency network [61, 62, 63]. ChIP-Seq analysis of TCF3 DNA binding sites, demonstrated a highly significant overlap with those of the pluripotency factors OCT4, NANOG and SOX2, indicating that Wnt pathway is physically connected to the core regulatory circuitry [61]. In mESCs, β-CATENIN possibly acts derepressing TCF3-specific targets by expelling Groucho/TLE

and the associated HDACs, which in the absence of Wnt signaling constitutively repress Wnt target genes [64]. Also, β-CATENIN counteracts TCF3-mediated repression of Lef1 resulting in the activation of target gene expression via LEF1—β-CATENIN complexes [65]. TCF1 and TCF3 have opposing roles in mESCs behavior since TCF1—β-CATENIN complex effectively inhibits TCF3 by competing for binding sites on chromatin [66]. Regarding the TCF/LEF independent role of β-CATENIN, it can promote pluripotency interacting with OCT4 and augmenting OCT4-dependent transcription [62]. Wnt essential role in ESC pluripotency is also supported by its contribution in enhanced efficiency of somatic cell reprogramming [67].

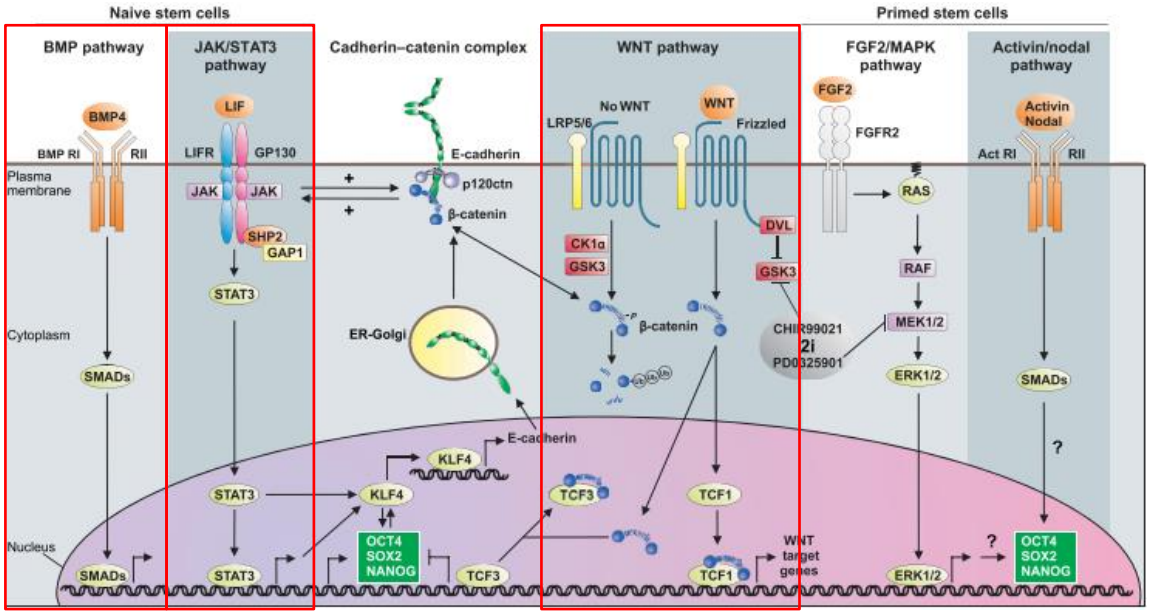


**Figure 2: Signaling pathways that promote mouse ESC pluripotency.** BMP, JAK/STAT3 and WNT signaling pathways govern mESCs pluripotency and self-renewal, up-regulating the expression of pluripotency factors and inhibiting differentiation genes [135].

## 3. ESC differentiation

Embryonic stem cells have the ability to self-renew and are able to spontaneously differentiate generating cellular derivatives of the endodermal, ectodermal, and mesodermal lineages under appropriate conditions in culture. The developing of differentiated lineages from ES cells provides a unique model system for studying the earliest steps of commitment in the context of development. Upon differentiation in vitro, ES cells form three-dimensional multicellular aggregates called embryoid bodies (EBs). EBs formation is a widely utilized differentiation protocol in vitro which is believed to recapitulate early mammalian embryogenesis [68]. EB structure mimic post-implantation embryo as it consists of an outer layer of the visceral endoderm and an internal epiblast-like cell mass which are able to differentiate into committed cell types including cardiomyocytes, skeletal muscles cells, endothelial cells, neuronal cells, adipocytes and haematopoietic precursors [68, 69] .

It is known that pluripotency maintenance and lineage specification are tightly linked. Actually, pluripotency factors are indispensable for ESCs multi-lineage commitment since it has been proposed that individual pluripotency factors are constantly prompting ESCs fate to their own lineage of interest while prohibiting commitment to the other lineages [70]. Specifically, the classical pluripotency factor OCT4 induces differentiation towards mesodermal lineage [19], SOX2 overexpression prompts neuroectodermal specification [71] and NANOG increased levels directs mesendodermal differentiation of human ESCs [72, 73]. Coexpression of the pluripotency circuity factors repress differentiation, however asymmetric expression can activate specific differentiation programs. Thus, continual extrinsic cytokine signaling such as LIF and BMP is essential for ESCs to remain undifferentiated ensuring the equilibrium of the key pluripotency factors [70].

Embryonic stem cells are characterized by a specific epigenetic landscape which involves activating histone modifications (H3K4me and H3K4ac) and DNA hypomethylation resulting in a “hyperdynamic” state of chromatin [74]. This open chromatin configuration renders it highly accessible to transcription factors maintaining ESCs pluripotency. However, silent developmental genes are bivalently marked by both active (H3K4me3) and repressive (H3K27me3) histone modifications,

indicating that these genes are primed for activation prior to ESC differentiation [75]. Polycomb (PcG), PRC1 and PRC2, and Trithorax (Trx)/ MLL group of proteins mediate the establishment of bivalent chromatin marks, H3K27me3 and H3K4me3 respectively, securing the ESC undifferentiated state but also developmental gene priming [76]. The transition between pluripotency and differentiation is accompanied by rapid and global changes in epigenetic and transcriptional profile. Upon differentiation, the open chromatin architecture is progressively replaced by a more restricted conformation limiting gene expression potential. Bivalent genes undergoing permanent repression are subjected to a process called "resolution", where more highly repressive heterochromatin associated histone modifications such as H3K9 and H3K27 methylation is expanded [74]. During this process, pluripotency network genes Oct4, Sox2, Nanog are actively repressed reinforcing the fact that epigenetic factors and the regulation of ESCs fate are strongly connected.

In the context of ESC differentiation, miRNAs play also important role mediating two main events of this process, reduction of proliferation rate and cell lineage commitment. During differentiation miRNAs levels alter and their expression is regulated by multi-layered regulatory processes including transcriptional, epigenetic, and/or post-transcriptional regulation [77]. The most abundantly expressed miRNAs in ESCs are the miR-290-295 and miR-302-367 clusters which are associated with pluripotency maintenance and cell cycle regulation [78]. Mir-290 cluster inhibits ESCs early differentiation and promotes high proliferation rate by assisting G1/S progression and regulation G2/M transition [79]. Mir-302 cluster is transcriptionally regulated by OCT4 and SOX2 and its expression also promotes G1/S transition, mediated by repression of cyclin D1 [80]. Upon differentiation, self-renewal promoting miRNAs are downregulated whereas differentiation-related miRNAs are upregulated such as let-7, the "antistemness" miRNA, which promotes cell cycle exit by repressing positive cell cycle regulators of G1/S phase and aiding G2/M progression [81]. Supporting that, miRNAs contributing to specification into the three germ layers are highly expressed, such as mir-338 and mir-340 clusters promoting definitive endoderm formation and mir-145 cluster positively regulating mesodermal and ectodermal differentiation [82]. Thus, it is apparent that miRNAs have a critical role in pluripotency maintenance and regulation of early mammalian development.

## 3.1 Neural differentiation of ESCs

In early development, nervous system patterning is divided into three main processes: neural induction, neurulation and regional specification. At the earliest steps in neural induction, neuroectodermal specification is considered as the default fate for epiblast cells, as a result of the inhibition of mesoderm and endoderm promoting signals such as Wnts, Nodal and BMPs [83]. In mammals, the node which is considered the cellular and molecular equivalent of Xenopus organizer, is responsible for the secretion of BMP, Wnt and Activin/Nodal signaling antagonists, along with the anterior visceral endoderm [84]. In vitro, ESC differentiation towards the neural lineage is accomplished by several established basic protocols which fall into three major categories; embryoid body-based, direct monolayer or co-culture [85]. In previous studies, efficient neuronal differentiation was accomplished using EB formation accompanied by RA treatment or co-culture with Pa6 bone marrow stromal cells [86, 87]. However, RA suppresses the expression of anterior genes and promotes caudalization, repressing the formation of rostral neural structures [87]. Alternatively, direct monolayer approach takes advantage of the "default" state of neuroectoderm development by culturing the ESCs in nutrient poor medium, limiting then cell-cell interactions and growth factor influence [88]. ESCs are able to generate neural stem cell (NSCs), the self-renewing and pluripotent population of the nervous system. NSCs have the properties of the embryonic radial glial and they can differentiate into all types of neural derivatives, neurons, astrocytes and oligodendrocytes [85].

In vitro differentiation of ESCs into neural progenitors and various types of neurons shares many mechanisms that govern neurogenesis during embryonic development. Critical signaling pathways such as Wnt, BMP and Acivin/Nodal are known to inhibit neuroectoderm formation during gastrulation but also ESC neuroectodermal specification in vitro [54, 86, 89, 90, 91, 92, 93]. Moreover, both processes have also structural similarities, since ESC-derived NSCs typically arrange themselves radically in a floral-like structure termed neural rosette which is comparable to the embryonic neural tube in terms of gene expression profile and functionality. This observation is enhanced by the fact that neural progenitors within neural rosettes are able to differentiate into the main classes of neuroepithelial progenitors in vivo

neurons, oligodendrocytes and astrocytes, suggesting that their differentiation is governed by similar mechanisms in vitro and in vivo [94, 95].

# 4. GRG5

## 4.1 The Gro/TLE family

*Drosophila groucho* (*gro*) allele was initially identified as a viable mutation (*gro*$^{1}$) that results in excessive bunches of sensory bristles above the adult eyes, a trait reminiscent of the bushy eyebrows of the American film comedian Groucho Marx [96]. *gro* is a member of the *Enhancer of split* [*E*(*spl*)] gene complex among the three structurally distinct types of gene products generated from this chromosomal region. *E*(*spl*)-C gene products act as regulators of the Notch signaling pathway and are involved in neurogenesis, wing development and eye morphogenesis [97]. Gro interacts with basic helix-loop-helix (bHLH) domain-containing transcription factors such as Hairy/ E(spl) (HES) family and functions as transcriptional corepressor via direct binding to their WRPW motif [98]. This interaction is required for the transcriptional repression of genes that direct three developmental processes in *Drosophila* : neurogenesis, embryo segmentation and sex-determination [99]. Loss-of-function mutations in *gro* cause hypertrophy of the nervous system due to lateral inhibition defect [100]. Since then, *groucho* homologs have been identified also in human and mouse named Transducin-Like Enhancer of split (TLE) and Groucho-related-gene (Grg) respectively. Grg/TLE family consists of more family members which are involved in biological processes including neurogenesis, gland and eye development and tumorigenesis.

## 4.2 Structural and functional characteristics

Grg/TLE proteins are divided into two distinct subgroups based upon their size, the long GRGs and the truncated family members. The long TLE/GRG subgroup consists of four pentadomain proteins (TLE1-4, GRG1-4), which, from N- to C-terminus are termed the Q, GP, CcN, SP and WD40-repeat domains. This five-domain structure is characterized by evolutionary conservation however among them Q and WD40 are most highly conserved whereas the three internal domains are less

conserved between the full-length members. Long TLEs/GRGs function as dedicated co-repressors for numerous transcription factors mainly due to their Q and WD40 domains. The glutamine-rich Q domain mediates tetramerization of long TLEs/GRGs members [101], which essential for their repressive function and interaction with TFs. GP domain, a region rich in glycine/proline is also critical for their repressive activity as it interacts with histone deacetylases (HDACs) and can modify chromatin conformation [102]. In the center of this variable region, CcN domain contains a nuclear localization signal (NLS), which regulates subcellular localization, and putative cdc2 kinase (cdc2) and casein kinase II (CKII) phosphorylation sites. The C'-terminal region with multiple Trp-Asp-repeat, WD40-repeat domain, facilitates protein-protein interactions and mediates transcriptional repression [97, 98, 103, 104, 105]. Grg/TLE proteins do not have the ability to bind to DNA by themselves, but they act as co-factors of different transcription factors and exert transcriptional effects.

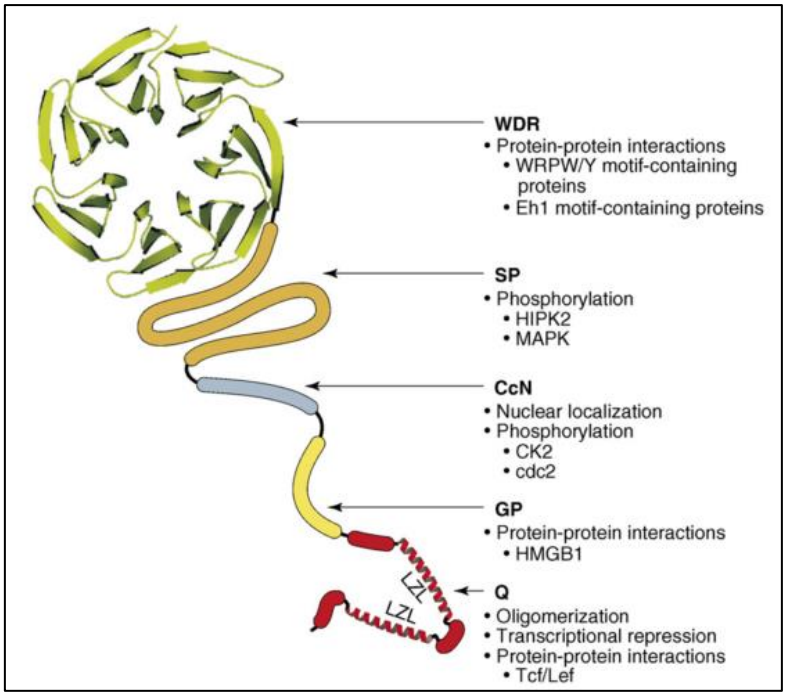


**Figure 3: The structure of Gro/TLE.** Representative structure of the pentadomain long TLEs/GRGs**.** They consist of WD40-repeat domain (green), SP (orange), CcN (blue), GP (yellow) and Q domain (red) [97].

Besides long TLEs/GRGs, there are also the truncated members which comprise the second subgroup of the family. The most representative members of this

subgroup is the Amino-terminal Enhancer of Split (AES) in humans and its mouse homolog GRG5 which are encoded by their own locus [104, 105 ,106, 107, 108]. AES/GRG5 consists only of the first two domains at the amino terminal, Q and GP. In addition, there exist truncated TLE/GRG family members which are alternatively spliced variants of the long TLE/GRG gene including GRG1-S and GRG3b [104, 105, 109]. AES/GRG5 has opposing function in comparison with long TLEs/GRGs since it can multimerize with them and hinder their repressive action due to its inability to interact with transcriptionally repressive HDAC proteins [110]. AES/GRG5 can antagonize transcriptional repression by long TLEs/GRGs by oligomerizing with them therefore losing their ability to interact with and repress certain TFs. Therefore, AES/GRG5 has been characterized as a dominant-negative TLE/GRG family member; however its biological function is context dependent and is not exclusively considered as a long TLE/GRG antagonist.

GRG5 is a 25kDa protein which has been reported to have nuclear, cytoplasmic or nucleocytoplasmic distribution in different cell types despite the lack of NLS containing CcN domain [107, 111, 112]. Therefore, it is believed that its localization depends on the intracellular position of its interacting partners. GRG5 heterodimerizes with full-length GRGs and interacts with transcription factors through the Q domain, destructing their repressive complexes. Therefore, GRG5 can antagonize the repressive function of long GRGs functioning subsequently as a de-repressor. Nevertheless, GRG5 can directly modulate transcription in a long GRGs independent manner since it can itself interact with transcription factors, thus acting either as co-repressor or co-activator [104, 105, 110, 113, 114]. Studies have been shown that GRG5 directly interacts with the androgen receptor (AR) and TFIIE, but also with the proapoptotic protein Bit1 modulating anoikis, a cellular process independent of transcription [110, 115]. Taken all these together, GRG5 is a multifunctional protein and this makes its study imperative in the future.

## 4.3 GRG5 in development and disease

GRG5 is broadly and constitutively expressed during development and adulthood with highest levels in muscles, heart and brain [106, 107, 112, 113, 116, 117]. GRG5 is precisely regulated since its over-expression in mice results in embryonic lethality in utero [114].

Contrary, GRG5 depletion does not cause any dramatic phenotype in mice since GRG5 null mice present postnatal skeletal growth defects but otherwise are viable [118]. Due to this effect of GRG5 deficiency on mice phenotype, the most extensively studied functional role of GRG5 is in growth and osteogenesis. *In vivo* GRG5 interacts with RUNX2, a transcription factor which is required for differentiation and proper function of osteoblasts, to regulate postnatal growth in mice. Moreover, GRG5 function downstream of RUNX2 and affects Ihh pathway which mediates bone and cartilage development [117, 118].

Furthermore, studies have been shown that GRG5 plays a critical role in early eye development through interaction with the transcription factors SIX3 and SIX6 by means of GST pulldown and yeast two hybrid systems [116, 119]. Also, the interaction between GRG5 and SIX3 is required for *Six3* transcriptional auto repression and this is essential for the modulation of SIX3-mediated retinogenic formation in postnatal mice [116].

As we stated above, long Gro/TLE family members have a well-established role in neurogenesis of many organisms. However, the role of GRG5 in human neural stem cells (NSCs) has been addressed recently [120]. GRG5 interacts with SOX2, a TF essential for neural stem cell self-renewal, and this interaction is required for the repression of many targets genes, promoting SOX2 antineurogenic activity [120]. GRG5 contribution in NSCs biology remains to be determined further.

GRG5 is also implicated in non-developmental processes including cancer and functions in a context dependent manner either as oncogene or as tumor suppressor. In acute myeloid leukemia (AML), *Grg5* is a target gene of AML1/ETO protein and up-regulate its expression. Thus, GRG5 enhances self-renewal of hematopoietic progenitor cells inhibiting their differentiation [121]. Conversely, GRG5 has been shown to inhibit metastasis of breast and colon cancer through regulation of RND3 expression [122] and suppression of Notch signaling [123] respectively. Taking everything into account, GRG5 is a multifunctional protein which is involved in numerous cellular/physiological processes and further studies are necessary to elucidate its biological function.

# Study objectives

Deciphering the mechanisms that govern ESCs pluripotency and neural differentiation will shed light to the processes by which ESCs establish their identity and regulate neural lineage commitment. Moreover, ESC neural specification has remarkable parallels to neurogenesis during embryogenesis, highlighting ESCs as model system to study nervous system development.

GRG5 is a multifunctional protein which is implicated in embryonic and postnatal developmental processes. Although previous studies have shown that GRG5 is a direct target of the pluripotency mediator STAT3, the functional role of GRG5 in ESCs remains to be determined.

The aim of this study is to elucidate the role of GRG5 in ESCs and its impact on ESCs neural differentiation potential.

Specifically, the aims of this project are:

- The evaluation of the effect of GRG5 depletion on ESC pluripotency.
- The analysis of the impact of GRG5 over-expression on ESC self-renewal.
- The investigation of GRG5 involvement in ESC neuroectodermal commitment.
- The unravelling of the molecular mechanism by which GRG5 influences neural specification.
- The establishment of an inducible GRG5 overexpression system for future studies on GRG5 role in neural fate determination and tumorigenesis.

# Materials and Methods

## 1. ESCs culture and differentiation

Feeder independent CGR8 murine ESCs were cultured on 0.2% gelatin in DMEM medium (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO), 0.2 mM β-mercaptoethanol (Applichem), 2 mM L-glutamine (GIBCO), 1× MEM nonessential amino acids (GIBCO), and 500U/ml LIF (ESGRO/Millipore).

KD and OE GRG5 cell lines were cultured on 0.2% gelatin in DMEM medium (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO), 0.2 mM β-mercaptoethanol (Applichem), 2 mM L-glutamine (GIBCO), 1× MEM nonessential amino acids (GIBCO), 500U/ml LIF (ESGRO/Millipore) and 1μg/ml puromycin and 300μg/ml G418 respectively.

For EB formation, cells were trypsinized and diluted in Iscove's Modified Dulbecco's Medium (IMDM; (Gibco) supplemented with 20% FBS (GIBCO) 0.2 mM β-mercaptoethanol (Applichem), 2 mM L-glutamine (GIBCO) and 1× MEM nonessential amino acids (GIBCO) to a final concentration of 1000 cells/20μl. EBs were cultured without LIF as hanging drops for 2 days and then were collected and cultured in suspension for 6 more days.

## 2. Plasmids construction

pMA2780-Gfp-Grg5 lentiviral vector was generated after the excision of Gfp-Grg5 coding sequence from pEGFP-Grg5 expression vector by NheI/BamHI digest and ligated in frame into XbaI/BamHI restriction sites of pMA2780 vector.

For MRed-SMAD1 and pBXGI-SMAD1 (SMAD1-GAL4) expression vectors, SMAD1 coding sequence was excised from pCDNA3-6myc-SMAD1 vector by EcoRI/XhoI digest. Then, Klenow protocol was performed for XhoI site to become blunt. Subsequently, EcoRI /blunt fragment ligated in frame into EcoRI/SmaI digested pDsRed-Monomer C2 and pBXGI vector respectively. Both plasmids express fused proteins under CMV promoter control.

## 3. Virus production

For the inducible cell line generation viruses were produced in HEK293T cells. Cells were plated in two 100mm dishes, in DMEM (GIBCO) supplemented with 10% FBS (GIBCO), 2mM L-glutamine (GIBCO) and 1mM Sodium Pyruvate (GIBCO). When the cells reached 60% confluency, one was transfected with the pMA2780-Gfp-Grg5 vector (20μg for 100mm dish), the packaging (12μg PsPAX2 for a 100mm dish) and envelope (6μg PMD2G for a 100mm dish) plasmids using the calcium phosphate protocol. The other dish was transfected with rtTA vector, the packaging and the envelope plasmids in the aforementioned quantities. The next day the medium was changed and 72h after transfection the supernatant was collected, centrifuged and filtered through a 0.45 μm filter.

## 4. Generation of inducible OE GRG5 ESC line

CGR8 ESCs were infected with lentivirus rtTA and selected with blasticidin (2μg/ml). A pool of resistant cells was generated. Blasticidin resistant cells were infected with pMA2780-Gfp-Grg5 lentivirus and selection was achieved with puromycin (2μg/ml). Selected clones were treated with 1 μg/ml doxycycline for 2 days and three independent clones with the highest GFP fluorescence were selected and cultured further.

## 5. Co-Immunoprecipitation Assay

Immunoprecipitation of the interacting protein complexes GRG5-SMADs was performed using HEK293T cells. Cells were transfected with GRG5-GFP and pCDNA3-6myc-SMAD1, SMAD5 or SMAD8 expression vectors. HEK293T transfected with GRG5-GFP and an empty pCDNA3-6myc were used as negative control. Cells were lysed in EBC buffer (50mM Tris PH 8, 170mM NaCl, 0.5% NP40, 50mM NaF) containing 1 mM PMSF. 200μg whole cell extracts was immunoprecipitated with anti-MYC and incubated with Protein-G agarose beads for 3h at 4oC. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-GRG5.

Immunoprecipitation of the interacting protein complex GRG5—B-CATENIN was performed using CONTROL and OE GRG5 cell line. KD GRG5 cells were used as negative control. Cells were lysed using the same conditions as mentioned above. 200µg whole cell extracts was immunoprecipitated with anti-GFP and incubated with Protein-G agarose beads for 3h at 4oC. Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-B-CATENIN.

## 6. Western Blotting

Cells were lysed in EBC buffer. Whole cell extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membranes using a transfer apparatus. Blocking was carried out by incubating the membranes in 4% BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60min. Membranes were incubated with primary antibodies diluted in blocking solution at 4°C overnight. They were then washed three times for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies at RT for 1 h. Blots were developed with the ECL system (Thermo Scientific).

## 7. Antibodies

Proteins were detected with primary antibodies presented in Table 1.

**Table 1:** Primary antibodies used to detect proteins for Western Blot analysis

| | |
|---|---|
| GRG5 | IMG-5408, Imgenex |
| ACTIN | sc-47778, Santa Cruz |
| OCT4 | sc-5279, Santa Cruz |
| NANOG | 8600S, Cell Signaling |
| SOX2 | 27485, Cell Signaling |
| NrOB1 | sc-13034X, Santa Cruz |
| B-CATENIN | sc-7963, Santa Cruz |
| GFP | B-2, Santa Cruz |

## 8. Quantitative RT-PCR

Total RNA was extracted using NucleoZOL reagent (Macherey-Nagel). 2µg RNA was reversely transcribed to cDNA by M-MLV Reverse Transcriptase (NEB) supplemented with RNase inhibitor according to the manufacturer's instructions. Quantitative RT-PCR was carried out using SYBR Green (Invitrogen) based detection and gene expression was normalized to *gabdh or actin.* The primers are presented in Table 2.

**Table 2:** Primer sequences for quantitative RT- PCR.

| | |
|---|---|
| Wnt3a FOR | 5' AAA GTG TAA ATG CCA CGG GTT 3' |
| Wnt3a REV | 5' GGG ACT CAG GGT GTT TCT C 3' |
| Pax6 FOR | 5' GGTGCTGGACAATGAAAACA 3' |
| Pax6 REV | 5' GGTACAGACCCCCTCGGATAA 3' |
| Id1 FOR | 5' GACTACATCAGGGACCTGCAGC 3' |
| Id1 REV | 5' GGCCGCCAAGGCACTGATCTCG 3' |
| Id3 FOR | 5' CCAGGTGGAAATCCTGCACC 3' |
| Id3 REV | 5' CTCTTGTCCTTGGAGATCACAA 3' |
| Sox17 FOR | 5' CTCTGCCCTGCCGGGATGG 3' |
| Sox17 REV | 5' AATGTCGGGGTAGTTGCAATA 3' |
| Eomes FOR | 5' GCTTCCGGGACAACTACGA 3' |
| Eomes REV | 5' GAGAGGAGGCCGTTGGTCT 3' |
| Map2 FOR | 5' AGCCGCAACGCCAATGGATT 3' |
| Map2 REV | 5' TTTGTTCCGAGGCTGGCGAT 3' |
| Bmp4 FOR | 5' TTCCTGGTAACCGAATGCT 3' |
| Bmp4 REV | 5' AAGTGTCGCCTCGAAGTC 3' |
| Tuj1 FOR | 5' AAGGTAGCCGTGTGTGACATC 3' |
| Tuj1 REV | 5' ACCAGGTCATTCATGTTGCTC 3' |
| Ngn2 FOR | 5' GCTGGCATCTGCTCTATTCC 3' |
| Ngn2 REV | 5' ATGAAGCAATCCTCCCTCCT 3' |
| Flk1 FOR | 5' GGATGGAGGCCTCTACACC 3' |
| Flk1 REV | 3' TGCCGACGAGGATAATGAC 3' |

| | |
|---|---|
| Gsc FOR | 5’ TGCTGCCCTACATGAACGTG 3’ |
| Gsc REV | 5’ CTCCAGGGCTTCGAGCTG 3’ |
| ErbB3 FOR | 5’ AAGGGTGTAAGGGACCAGAA 3’ |
| ErbB3 REV | 5’ AGTAGCGTCTCATAGCCCTT 3’ |
| Talin FOR | 5’ GCAGTCTCCGCTACCATAGA 3’ |
| Talin REV | 5’ ATGGCTCAAATTGCATCGTC 3’ |
| Tcf7l1 FOR | 5’ TCTGGATGAGGTCAAGTCGT 3’ |
| Tcf7l1 REV | 5’ GGGCCATTTCATCTGTAGG 3’ |
| Thbs1 FOR | 5’ AGAGCATCTTCACCAGGGAT 3’ |
| Thbs1 REV | 5’ ATGTAGTTGGTGCGGATAGC 3’ |
| Tcf7l2 FOR | 5’ CATCCGCTAGGATGGTTAGT 3’ |
| Tcf7l2 REV | 5’ AGTCCTGATGCTTTGAGCTG 3’ |
| Gata3 FOR | 5’ GATGTAAGTCGAGGCCCAAG 3’ |
| Gata3 REV | 5’ ATTAGCGTTCCTCCTCCAGA 3’ |
| Igf2 FOR | 5’ GGAAAACGACTGGGCATTG 3’ |
| Igf2 REV | 5’ CCAAAGAGATGAGAAGCACCA 3’ |
| Porcn FOR | 5’ CTTCGCAAGTGGCTACGAG 3’ |
| Porcn REV | 5’ AGGCGAAGGGCATTTTTGA 3’ |
| Smyd1 FOR | 5’ GTGGACACGTTCTTGCAGTA 3’ |
| Smyd1 REV | 5’ CGGAGCTCAATCTTGCCATT 3’ |
| Gapdh FOR | 5’ CTCCTCAGACCGCTTTTTG 3’ |
| Gapdh REV | 5’ TCCTCGGCATAATGATTAGG 3’ |
| Grg5 FOR | 5’ AGAAGTCAGAGATGCAGAGGC 3’ |
| Grg5 REV | 5’ GGAGCTGCTGTCGGATGA 3’ |
| Actin FOR | 5’ GTGTGACGTTGACATCCGTA 3’ |
| Actin REV | 5’ GTAACAGTCCGCCTAGAAGC 3’ |

## 9. Luciferase Assays

Cells were transfected with BRE-Luc reporter plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) and were stimulated 24h later using 500units/ml LIF (ESGRO/Millipore), 2μM CHIR99021 (Selleckhem) and 80ng/ml BMP4 (R&D) respectively. Hes1-Luc and Hes5-Luc reporters were used to measure Notch signaling activity. Cells were co-transfected with NICD expression plasmid to activate Notch signaling. Cells were co-transfected with the 5xGAL4UAS luciferase reporter and a SMAD1-GAL4 expression vector to detect the transcriptional activity of SMAD1. Luciferase activity was assayed 48h upon transfection and was normalized using β-galactosidase reporter assay.

## 10. Transwell Assay

ESCs were suspended in 0% FBS ESC culture media containing 0.1% BSA (GIBCO) and 1 μM Retinoic Acid (SIGMA) and plated at $0.15_X10^6$ cells/ml on a 8 μm pore size membrane. The lower compartment was filled with 10% FBS containing ESC media. After 35hr incubation, the transwell insert was removed and placed into 4% paraformaldehyde for 10min. Cells were washed with dH2O to remove paraformaldehyde. Using a cotton swap cells were scraped off the top of the transwell insert. Afterwards, cells were treated with a staining solution of 10μg/ml DAPI, 0.5% Triton X-100 in 1x PBS, for 10-15min. Transwell insert was removed and washed with 1x PBS. Migrated nuclei were visualized and counted under fluorescent microscope.

## 11. ESC Karyotyping

For karyotype analysis ESCs were seeded on gelatin in ESC medium. When cells became 70% confluent the medium was changed and 2h later they were treated with 20ng/ml Demecolcine (Sigma D6279) to synchronize cells in metaphase. After 5h cells were trypsinized, incubated with hypotonic solution for 6min, fixed (3Methanol: 1Acetic Acid) and dropped on acid washed slides.

## 12. Live Imaging

For GRG5-SMAD1 colocalization, HEK293T cells were seeded in a 4-well chamber (Lab-Tek®). After 24h, cells were transfected with Grg5-gfp and Smad1-mRed expression vectors. Gfp expression vector was used as negative control. Cells were stimulated 24h later using 80ng/ml BMP4 (R&D). The next day samples were incubated with DRAQ5 in medium for 10min in 37°C and visualized under confocal microscope (Biorad).

## 13. Statistical analyses

Student's t-test was used for all statistical analyses. Statistical significance was defined as follows: * means $p < 0.05$. Values were presented as the mean ± SD.

# Results

## 1. Role of GRG5 in ESC self-renewal and differentiation

### 1.1 GRG5 depletion destabilizes ES cell pluripotent state

In previous work of our laboratory, two KD Grg5 stable cell lines where generated using two distinct Grg5 specific shRNA-Plko.1 lentiviral vectors (Sigma Aldrich, SHCLNV-NM_010347, TRCN0000097716, TRCN0000097717). In agreement with the data obtained with TRCN0000097716 Grg5 shRNA, here we present that ESCs KD Grg5 using TRCN0000097717 shRNA similarly are characterized by down-regulation of crucial pluripotency factors including SOX2, OCT4 and NANOG (Fig.1A). Moreover, representative developmental factors of all three germ layers were concomitantly de-repressed at the mRNA level (Fig.1B). Moreover we also validated previous RNA-seq analysis using q-PCR for selected genes (Fig.1C).

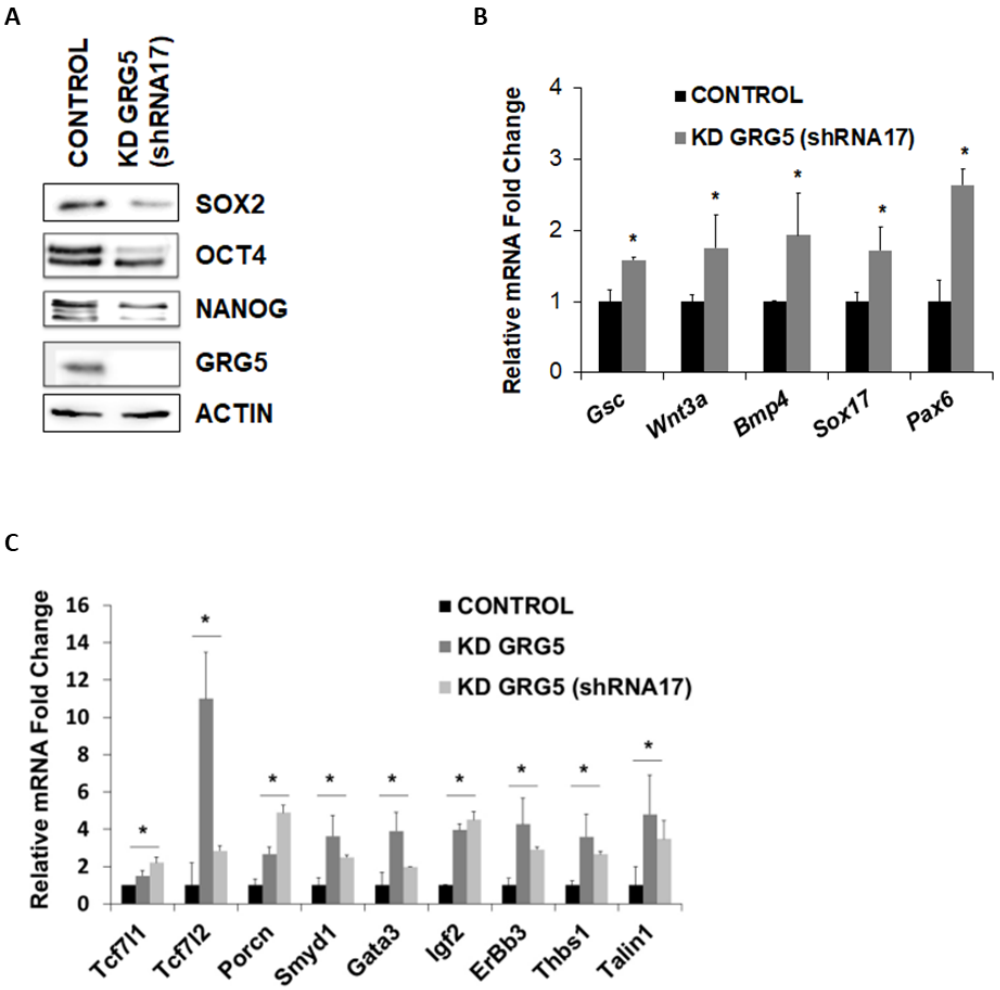

**Figure 1: GRG5 knock-down destabilizes cell pluripotent state.** (A) Protein levels of pluripotency factors upon depletion of GRG5. (B) Relative mRNA levels of differentiation markers in CONTROL and KD GRG5 ESCs. mean + SD of n=3 independent experiments. *P<0.05. (C) Validation of RNA-sequencing results. qPCR analysis of differentially expressed genes in CONTROL and both KD GRG5 ESC lines. mean + SD of n=3 independent experiments. *P<0.05.

## 1.2 Forced expression of GRG5 enhances ESC stemness and their proliferation and migration potential

Previous report has showed that strong transgenic GRG5 expression leads to mice embryonic lethality 114 so accordingly we questioned the impact of GRG5 overexpression on ESC phenotype. The already generated stable overexpressing GRG5-GFP clone #7 (OE GRG5 ESCs) presents significantly higher protein level of GRG5. Overexpression of GRG5 results in increased protein levels of key pluripotency factors including SOX2, NANOG and NROB1 (Fig.2A). Moreover, growth rate analysis of OE GRG5 ESCs revealed impressively increased proliferative potential of these cells (Fig.2B). Based on previous work of our laboratory which has shown that intramuscularly and intraperitoneal injection of OE GRG5 cells causes the formation of malignant teratomas with strong invasion capacity, we were prompted to study OE GRG5 ESCs in more detail for additional tumor cell like properties. We used in vitro transwell migration assay to analyze their migration potential upon induction of differentiation with RA treatment. The percentage of cells that migrated toward a chemo-attractant gradient was measured 24h upon plating and was approximately three times higher in case of OE GRG5 ESCs (Fig.2C). To exclude the possibility of chromosomal abnormalities that are often linked with increased ESC tumorigeniticity 124, we performed karyotype analysis which did not show differences in OE GRG5 ESCs as compared to the WT (Fig.2D).

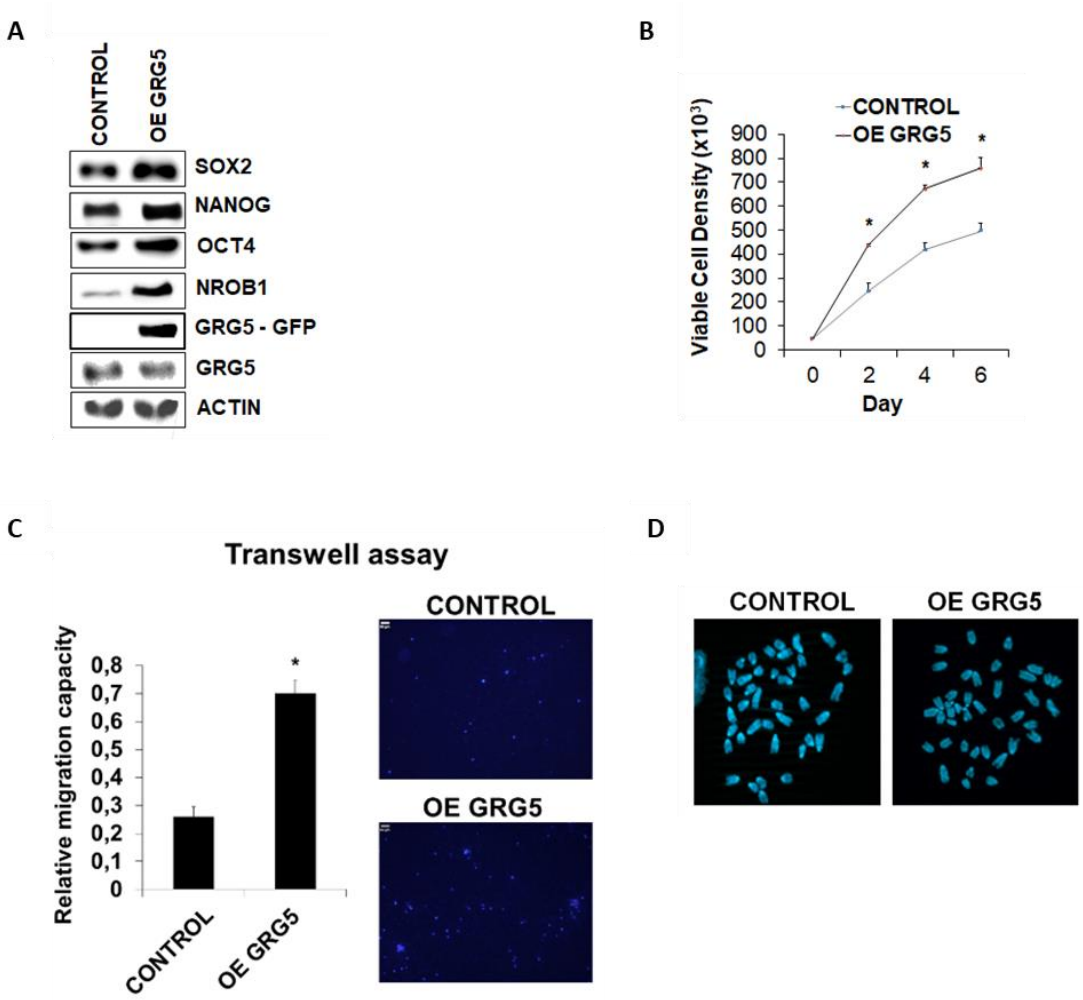


**Figure 2: Forced expression of GRG5 enhances ESC proliferation and migration capacity.** (A) Western Blot showing expression of pluripotency markers in CONTROL and OE GRG5 ESCs. (B) Growth curves of CONTROL and OE GRG5 ESCs during in vitro culture. mean ± SD of n=3 independent experiments. (C) Transwell assay showing the elevated migration capability of OE GRG5 ESCs. Quantification and photos of the DAPI stained nuclei 24h upon plating. mean + SD of n = 3 independent experiments. *P<0.05 (Scale bar, 100µm). (D) Karyotype analysis. Both CONTROL and OE GRG5 ESCs present intact chromosome number.

## 1.3 GRG5 influences ESC differentiation decisions in favor of neuroectoderm

Then we evaluated the impact of GRG5 depletion in ESC differentiation potential through Embryoid bodies (EBs) formation. In line with the RNA-seq results, qPCR analysis of representative differentiation factors revealed that the KD GRG5 EBs are characterized by higher induction of mesendodermal genes (*Wnt3a, Flk1, Eomes, Gata4*) (Fig.3B). In contrast, they express lower amounts of neuroectodermal markers (*Ngn2, Pax6, Map2, Tuj1*) (Fig.3B).

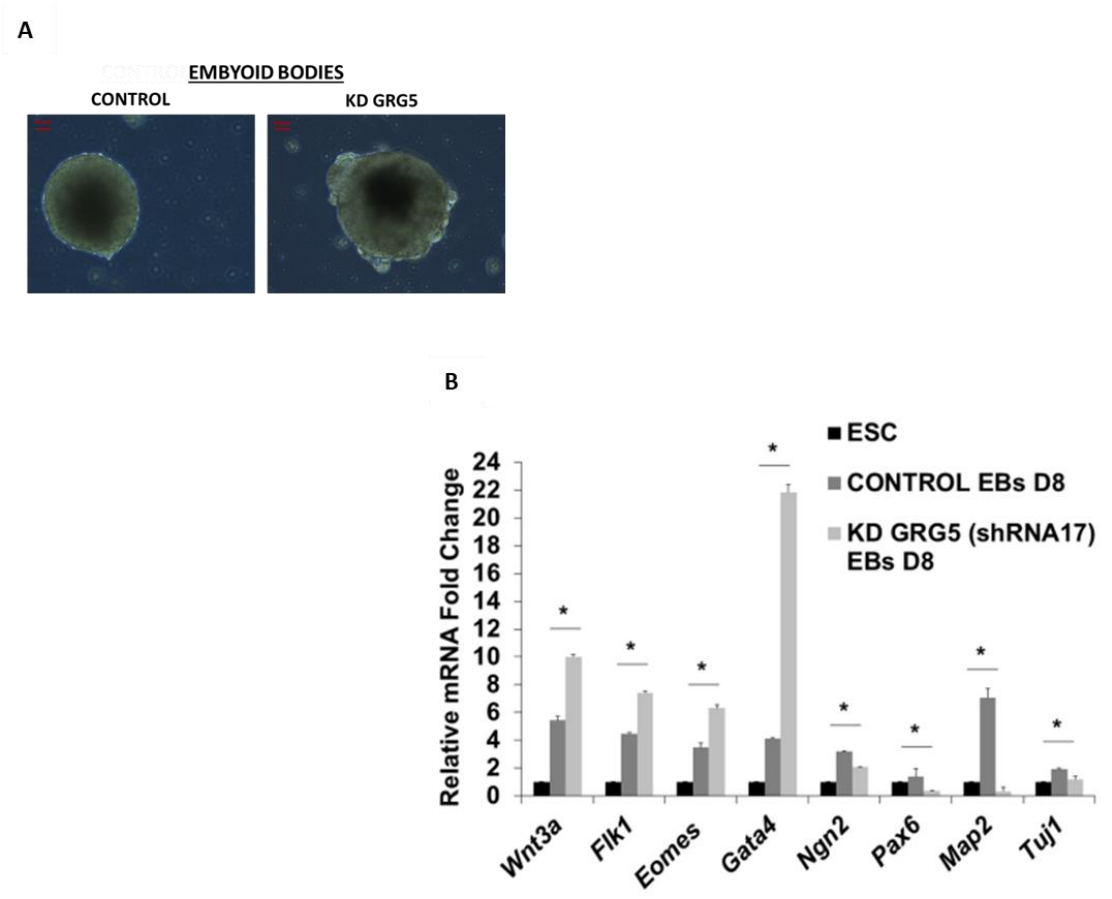


**Figure 3: GRG5 depletion impedes ESCs neuroectodermal specification.** (A) Photos showing CONTROL and KD GRG5 EBs at Day 8. (Scale bar, 50µm) (B) qPCR analysis of mesodermal (*Wnt3a, Flk1, Eomes*), endodermal (*Gata4*) and neuroectodermal (*Ngn2, Pax6, Map2, Tuj1*) markers upon differentiation of CONTROL and KD GRG5 ESCs through EBs formation at Day 8. mean + SD of n=3 independent experiments. *P<0.05.

## 2. Role of GRG5 in ESC neuroectodermal commitment

### 2.1 GRG5 interacts with SMADs and down-regulates Bmp pathway thus promoting ESCs neural specification

As we described above, GRG5 positively regulates SOX2 expression, one of the master regulators of pluripotency. Except of this role, SOX2 is also a neuroectoderm lineage specifier with important role in neurogenesis 125, 126. Moreover, Bmp as well as Wnt signaling is known for its anti-neurogenic activity, inhibiting ESCs neural differentiation 127. Based on a previous study which has shown that GRG5 represses Wnt signaling in human cells and zebrafish embryos 128, we were prompted to examine GRG5 function in neuroectodermal commitment of ESCs.

Initially, we assessed the effect of GRG5 depletion and overexpression on the activity of BMP pathway in mouse ESCs, using Luciferase assay and target gene expression analysis. CONTROL, KD (#16) and OE GRG5 ESCs were transfected with BRE-LUC reporter plasmid to monitor BMP pathway. Remarkably, luciferase activity measurement showed that GRG5 represses Bmp pathway, upon stimulation with BMP4 (Fig.4A). Accordingly, expression analysis of BMP targets Id1 and Id3 confirmed reduction in pathways activity upon GRG5 over-expression (Fig.4B).

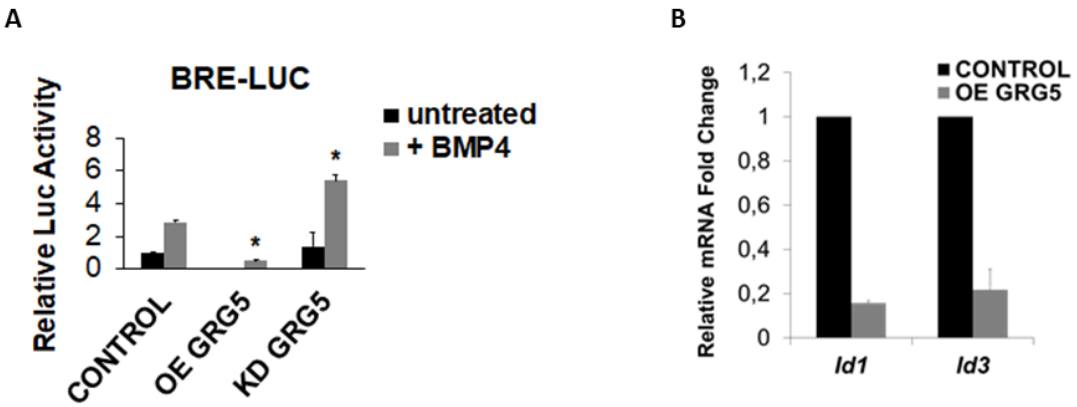


**Figure 4: GRG5 down-regulates BMP signaling pathways in ESCs.** (A) Luciferase assay to monitor BMP signaling in CONTROL, OE GRG5 and KD GRG5 ESCs. mean + SD of n = 3 independent experiments. *P<0.05. (B) Relative mRNA level of BMP pathway target genes Id1 and Id3 in CONTROL and OE GRG5 ESCs.

To further investigate the molecular mechanism behind GRG5 repressive activity on Bmp pathway, we questioned if GRG5 interacts with the Bmp pathway mediators SMAD1, SMAD5 and SMAD8 and for this reason we performed co-immunoprecipitation assays in HEK293 cells. We discovered that GRG5 interacts with SMAD1 and SMAD5 but not with SMAD8 (Fig.5A). To gain insight into the functional significance of GRG5 and SMAD1 interaction, we employed the GAL4-UAS luciferase system. The activity of the luciferase reporter was tested upon expression of a fused SMAD1-GAL4 protein in the presence/absence of GRG5. As it is shown in figure 5B, overexpression of GRG5 inhibited the transcriptional activity of SMAD1. In support of this observation, we visualized GRG5 and SMAD1 localization prior and upon BMP4 stimulation. GRG5 as well as SMAD1 has mainly cytoplasmic localization before the stimulation of Bmp pathway as it is expected. On the contrary, upon stimulation SMAD1 obtains nuclear localization as well, and GRG5 seems to follow the same distribution. In both conditions, it is obvious that GRG5 and SMAD1 co-localize supporting our finding about GRG5-SMAD1 interaction (Fig.5C). All the above data suggest a repressive mechanism of action in which GRG5 interacts with SMADs and modulates their transcriptional activity.

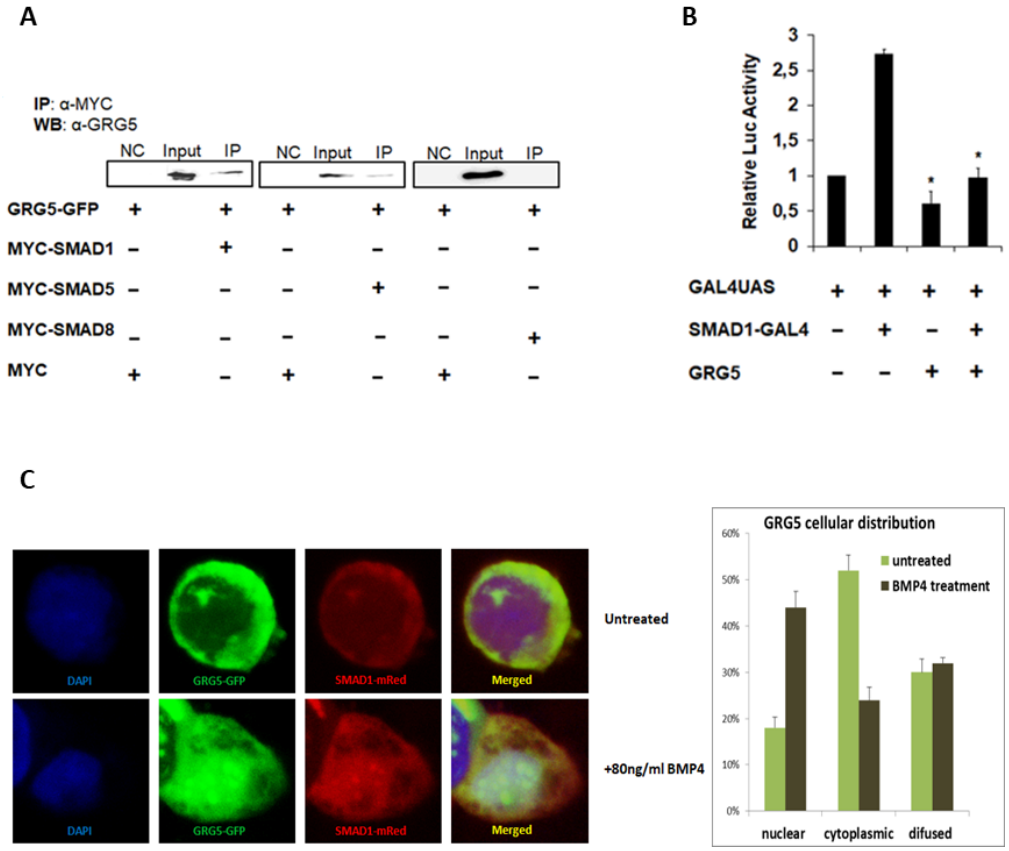

**Figure 5: GRG5 interacts with SMAD1 and SMAD5 and hinders SMAD1 transcriptional activity.** (A) Co-immunoprecipitation assay showing interaction of GRG5 with SMAD1 and SMAD5 but not with SMAD8. Whole cell lysates of HEK293 cells, transfected with GRG5-GFP and MYC-SMAD1 or MYC-SMAD5, was immunoprecipitated with anti-MYC and immunoblotted with anti-GFP. HEK293 cells transfected with MYC and GRG5 GFP were used as negative control NC. (B) Lusiferase assay using the GAL4UAS system to show the transcriptional activity of SMAD1 upon transient overexpression of GRG5 in HEK293 cells. mean + SD of n = 3 independent experiments. *P<0.05. (C) SMAD1 and GRG5 co-localization and nuclear translocation of both upon Bmp4 induction. In each condition at least 100 cells were analyzed. mean + SD of n = 3 independent experiments.

## 2.2 GRG5 interacts with B-CATENIN in OE GRG5 cells and down-regulates Wnt pathway

Previous studies have shown that Wnt and BMP signals have negative effects on mammalian neural differentiation. Earlier findings of our laboratory have demonstrated that GRG5 overexpression reduces the activity of Wnt pathway using luciferase activity measurement upon stimulation with Wnt agonist CHIR. Accordingly, expression analysis of Wnt downstream targets T-brac and Axin2 confirmed the former observation (data not shown). In addition, as we stated above, GRG5 is considered as a positive regulator of neural fate commitment. To shed light on the molecular mechanism behind GRG5 repressive activity on Wnt pathway, firstly we examined the expression levels of β-CATENIN in CONTROL, KD GRG5 and OE GRG5 cell lines (Fig.6A). Unexpectedly, in OE GRG5 cells β-CATENIN was upregulated although we have previously shown that in these cells the Wnt pathway is repressed. To further examine this unorthodox accumulation of β-CATENIN, we question if GRG5 interacts with β-CATENIN and for this reason we performed co-immunoprecipitation assays in CONTROL and OE GRG5 ESCs (Fig.6B). We discovered that GRG5 interacts with β-CATENIN in OE cells (Fig.6Bi).

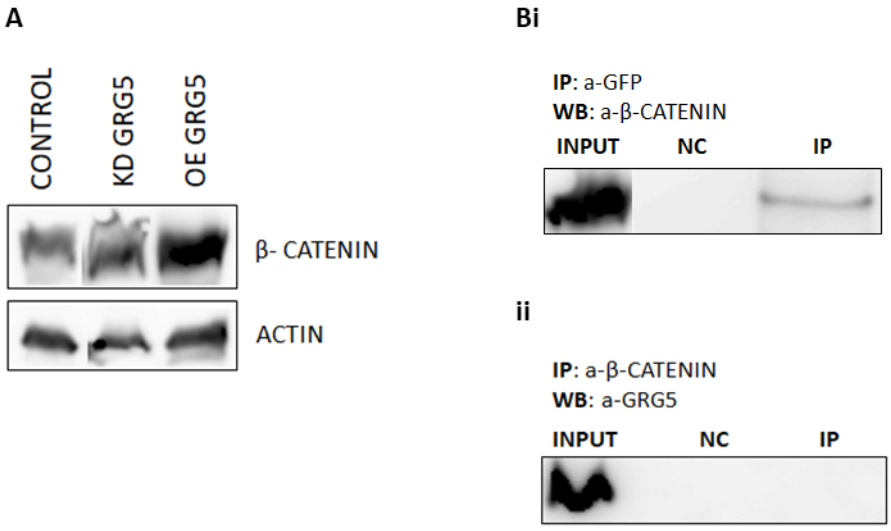


**Figure 6: GRG5 interacts with β-CATENIN in OE GRG5 cells.** (A) Western blot for β-CATENIN in CONTROL, KD and OE GRG5 ESCs. (B) (i) Co-immunoprecipitation assay showing no interaction of β-CATENIN with GRG5 in CONTROL cells. Whole lysate of CONTROL cells was immunoprecipitated with anti-β-CATENIN and immunoblotted with anti-GRG5. KD GRG5 cells were used as negative control NC. (ii) Co-immunoprecipitation assay showing interaction of β-CATENIN with GRG5 in OE cells. Whole lysate of OE cells was immunoprecipitated with anti-GFP and immunoblotted with anti-β-CATENIN. KD GRG5 cells were used as negative control NC.

## 2.3 Stable ESC line generation for regulated overexpression of GRG5

Previous experiments of our laboratory have shown that GRG5 is required for successful ESC specification towards neuroectoderm. It has been demonstrated through monolayer differentiation approach and RA induced neural differentiation that GRG5 depletion severely reduced the percentage of both neural progenitor cells (NPCs) and differentiated neurons whereas overexpression of GRG5 accelerated neural commitment. Taking this into account and in order to gain insight into the function of GRG5 in neural differentiation, we set out to generate a stable ESC line for inducible overexpression of GRG5. This will give us the advantage of regulated overexpression of GRG5 so as to assess its impact on the different stages of neural differentiation *in vitro*.

Initially, we constructed the lentiviral expression vector for this inducible system placing GFP-GRG5 coding sequence under the control of TetO elements (**T**etracycline **R**esponse **E**lement) using molecular cloning technics. The aforementioned system is referred to the Tet-On system meaning that there is the need of the *reverse tetracycline transactivator protein (rtTA)* which is capable of binding the TRE operator only if bound to tetracycline or one of its derivatives, such as doxycycline, and thus activate transcription. For this reason firstly we generated a stable rtTA "mother" line by infecting ESCs with rtTA lentivirus and pools of infected cells were selected with blasticidin. Afterwards, these cells were infected with lentivirus produced by the abovementioned vector, carrying the Gfp-Ggr5 sequence. Selection of five independent clones was performed with puromycin. All of them were examined for their efficiency of producing the recombinant protein after 1μg/ml doxycycline treatment.

Here we present the analysis of clone #5 which presented the higher efficiency in terms of expression. OE GRG5 #5 exhibits almost five fold enhanced Grg5 mRNA levels and increased expression levels of pluripotency factors in consistency with the constitutive OE GRG5 line.

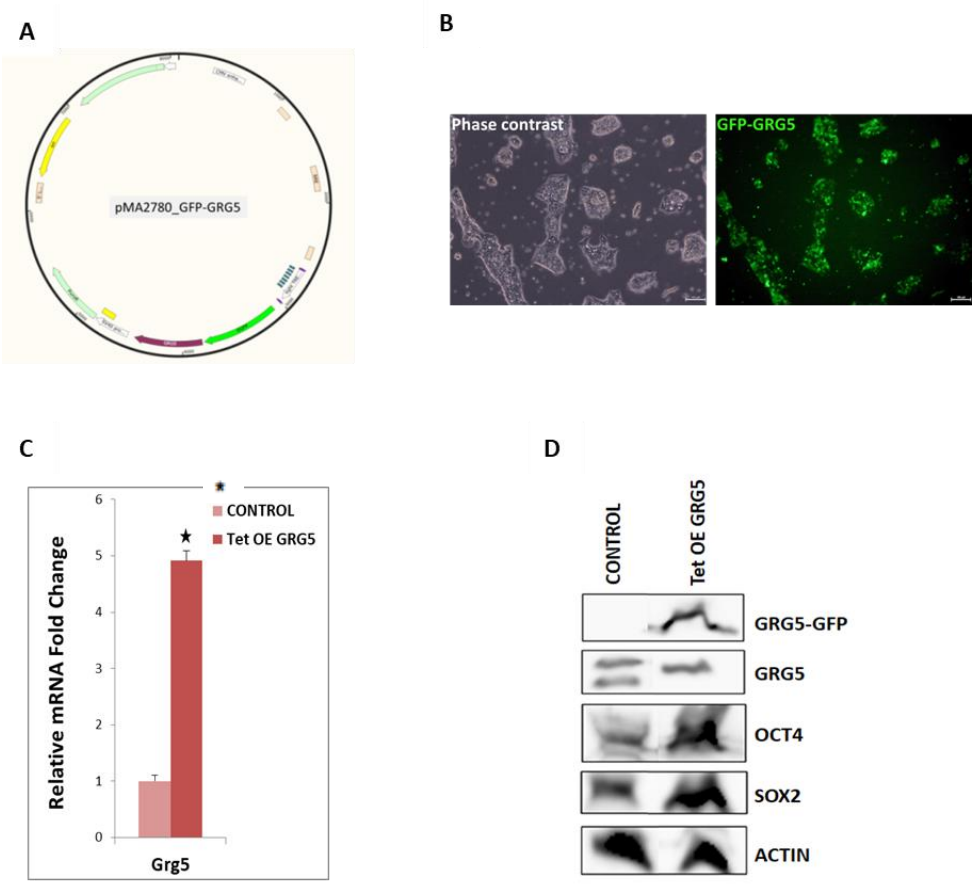

**Figure 7: Tetracycline regulated OE GRG5 clone #5 analysis.** (A) Structure of GFP-GRG5 lentiviral plasmid for controlled expression. (B) GFP fluorescence of clone #5 after 2 days of 1µg/ml doxycycline treatment. (Scale bar: 100µm) (C) Relative mRNA level of Grg5 in CONTROL and OE GRG5 #5 ESCs. mean +SD of n=3 independent experiments. *P<0.05. (D) Western Blot showing expression of pluripotency markers in CONTROL and OE GRG5 # 5 ESCs.

# Discussion

ESCs, the progeny of the inner cell mass (ICM) of the blastocyst, are characterized by an almost unlimited developmental potential, are able to remain pluripotent and can differentiate into derivatives of all three germ layers *in vitro* and *in vivo*. The proliferative and developmental potential of ESCs has rendered them as key cellular tool in basic research and introduced a new era of regenerative medicine. The balance between self-renewal and differentiation is secured by a combination of extrinsic signaling molecules and a complex regulatory network of transcription factors [129]. Over the past years more and more studies has been focused on elucidating the molecular regulation of ESC pluripotency and consequently have paved the way for ESC applications.

In this research project, we examined the role of GRG5 in embryonic development using mESCs as a model of study. We establish its function as a critical component of the regulatory network of ESC pluripotency and its involvement in ESC differentiation, especially in neuroectodermal commitment.

GRG5 is a direct target of STAT3 in ESCs and hence contributes to the positive effect of STAT3 on self-renewal. Moreover, GRG5 acts by de-repressing Tcf-mediated transactivation and thus can regulate a crosstalk between LIF/STAT3 and Wnt signaling [130]. In the present study we demonstrate that GRG5 acts as regulator of stem cell pluripotency since depletion of GRG5 deregulates the pluripotent state by downregulating the core pluripotency factors and de-represses a variety of developmental markers hinting ESC exit from pluripotency.

On the contrary, GRG5 forced expression in ESCs results in upregulation of key pluripotency factors and increased proliferation rate. In addition, OE GRG5 ESCs present enhanced migration potential *in vitro* which is consistent with previous findings of our laboratory that intramuscularly and intraperitoneal injection of OE GRG5 cells in mice causes the formation of malignant teratomas with strong invasion capacity *in vivo*. To ensure that this phenomenon is not attributed to chromosomal abnormalities, which are usually linked with increased ESC tumorigeniticity [124], we performed karyotype analysis that revealed no genetic aberrations. Recently has been shown that TLE3 and 4, two full length members of the Gro/TLE family, repress

the ESC transcription factor network and promote differentiation [131]. Conversely, here GRG5 exhibits an inverse function pattern which possibly is accredited to the antagonism between GRG5 and long GRGs, inhibiting the exit from pluripotent state.

Apart from a role in the regulation of pluripotency, the data of this study show that GRG5 is also involved in ESC linage commitment. As we stated above, RNA-seq data have indicated that in the absence of GRG5 most of the upregulated genes are implicated in mesoendodermal differentiation along with the enhancement of components of Wnt and Bmp pathway. Especially, Tcf7l1 (or Tcf3) which its repressive activity is required for mesoderm specification in response to Wnt stimulation [132], is notably increased when GRG5 is absent. In line, GRG5 depleted EBs are characterized by higher induction of mesoendodermal genes whereas neuroectodermal markers are downregulated. This fact reveals that GRG5 influences ESC differentiation decisions in favor of the neuroectoderm and thus is required for the neuroectodermal commitment of ESCs.

Bmp and Wnt signaling are known for inhibiting ESCs neural differentiation and thus being anti-neurogenic signaling cascades [92]. In the present study we describe for the first time that overexpression of GRG5 results in the repression of Bmp signaling, by uncovering a physical interaction between GRG5 and the pathway mediators SMAD1 and SMAD5 which is inhibitory for their transcriptional activity. GRG5 was previously found to antagonize Wnt/β-catenin signaling in human cells and zebrafish embryos serving as a TCF4/Tcf7l2 corepressor [128]. In agreement, data of our laboratory showed that GRG5 has an inhibitory effect on Wnt upon its overexpression (data not shown). The unexpected accumulation of β-CATENIN in OE cells possibly is partially attributed to the downregulation of *Axin2* (data not shown). *Axin2* expression is induced via the β-CATENIN/Tcf pathway thus being a direct downstream target and its observed downregulation is expected since Wnt signaling is suppressed in OE GRG5 cells. Besides, AXIN2 is a component of the destruction complex and may assist the downregulation of β-CATENIN to normal levels after Wnt stimulation, forming a negative feedback loop [133]. It is plausible that Axin2 transactivation by β-CATENIN is impaired due to GRG5/β-CATENIN interaction. Thus, *Axin2* is not adequately expressed, thus the negative feedback loop is quenched leading to inefficient degradation of β-CATENIN and its accumulation. Except from β-

CATENIN degradation as regulatory mechanism, it has been shown that Wnt pathway is also negatively regulated in the nucleus by β-CATENIN binding to inhibitory proteins including ICAT in *Xenopus* [134]. We suppose that the interaction of β-CATENIN with GRG5 inhibits the interaction of β-CATENIN with TCF-3 thus repressing β-CATENIN-TCF3-mediated transactivation. The exact molecular mechanism by which GRG5 function in Wnt signaling cascade in OE cells remains to be determined. On the whole, we ascribe the neurogenic function of GRG5 to its potency to repress the main anti-neurogenic pathways.

Preexisting results have also shown that depletion of GRG5 severely hindered the generation of both neural progenitor cells (NPCs) and differentiated neurons, upon monolayer differentiation approach, indicating that GRG5 is required for successful ESC specification towards neuroectoderm. In contrast, overexpression of GRG5 accelerated neural commitment, yielding higher percentage of both SOX1+ NPCs and TUJ1+ neurons by the end of the differentiation process. Impressively, OE GRG5 ESCs maintained higher neural specification potential compared to CONTROL even under BMP and Wnt activation (data not shown), verifying our hypothesis that GRG5 is a positive regulator of ESC neural differentiation. Collectively, GRG5 promotes ESC neural differentiation through suppression of Wnt and BMP by interacting with the pathways mediators. The fact that GRG5 positively regulates SOX2, a genuine neuroectoderm lineage specifier [125, 126], may contribute to its pro-neural activity as well.

In light of our previous findings and in order to elucidate the functional role of GRG5 in in cell growth control and neural differentiation, we established an inducible OE GRG5 cell line. Upon doxycycline treatment, these cells have similar expression pattern with the constitutive OE GRG5 cell line, exhibiting increased levels of pluripotency factors. *In vitro*, differentiation approach experiments will provide a good opportunity to assess GRG5 impact on the different stages of neural differentiation and also on the differentiation potential of ESCs into the main classes of NPCs, neurons, oligodendrocytes and astrocytes. Moreover, taking into account that GRG5 overexpression in transgenic mice causes embryonic lethality [114], this regulated system will be more relevant in a prospective *in vivo* study of GRG5 OE on tumorigenesis and brain development.

# References


1. Hillman, B. N., William, S. & Road, S. P. The effect of spatial arrangement on cell determination during mouse development. *J. Embryol. Exp. Morphol.* **28,** 263–278 (1972).
2. Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7634–7638 (1981).
3. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154 (1981).
4. Kooreman, N. G. & Wu, J. C. Tumorigenicity of pluripotent stem cells : biological insights from molecular imaging. *J. R. Soc. Interface* (2010).
5. Nishikawa, S., Jakt, L. M. & Era, T. Embryonic stem-cell culture as a tool for developmental cell biology. *Nature* **8,** 502–507 (2007).
6. Brons, I. G. M. *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448,** 191–196 (2007).
7. Tesar, P. J. *et al.* New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448,** 2–8 (2007).
8. Nichols, J. & Smith, A. Naive and Primed Pluripotent States. *Stem Cell* **4,** 487–492 (2009).
9. Guo, G. *et al.* Naive pluripotency stem cells derived directly from isolated cells of the human inner cell mass. *Stem Cell Reports* **6,** 437–446 (2016).
10. Guo, G. *et al.* Epigenetic resetting of human pluripotency. *Development* 2748–2763 (2017). doi:10.1242/dev.146811
11. Chambers, I. *et al.* Functional Expression Cloning of Nanog , a Pluripotency Sustaining Factor in Embryonic Stem Cells. *Cell* **113,** 643–655 (2003).
12. Mitsui, K. *et al.* The Homeoprotein Nanog Is Required for Maintenance of Pluripotency in Mouse Epiblast and ES Cells. *Cell* **113,** 631–642 (2003).
13. Nichols, J. *et al.* Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4. *Cell* **95,** 379–391 (1998).
14. Boyer, L. A. *et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122,** 947–956 (2005).
15. Yeo, J. & Ng, H. The transcriptional regulation of pluripotency. *Cell Res.* **23,** 20–32 (2013).
16. Rosner, M. H. *et al.* A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345,** 686 (1990).
17. Scholer, H. R., Dressler, G. R., Rohdewohid, H. & Gruss, P. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J.* **9,** 2185–2195 (1990).
18. Loh, Y. *et al.* The Oct4 and Nanog transcription network regulates pluripotency

in mouse embryonic stem cells. *Nat. Genet.* **38,** 431–440 (2006).

19. Niwa, H., Miyazaki, J. & Smith, A. G. Quantitative expression of Oct-3 / 4 defines differentiation , dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24,** 2–6 (2000).
20. Shimozaki, K., Nakashima, K., Niwa, H. & Taga, T. Involvement of Oct3 / 4 in the enhancement of neuronal differentiation of ES cells in neurogenesis-inducing cultures. *Development* 2505–2512 (2003). doi:10.1242/dev.00476
21. Kamachi, Y., Uchikawa, M., Kondoh, H. & Biology, C. Pairing SOX off with partners in the regulation of embryonic development. *Trends Genet.* **9525,** 2–7 (2000).
22. Wood, H. B. & Episkopou, V. Comparative expression of the mouse Sox1 , Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech. Dev.* **86,** 197–201 (1999).
23. Avilion, A. A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 126–140 (2003). doi:10.1101/gad.224503.derm
24. Masui, S. *et al.* Pluripotency governed by Sox2 via regulation of Oct3 / 4 expression in mouse embryonic stem cells. *Nat. Cell Biol.* **9,** (2007).
25. Zhang, S. & Cui, W. Sox2 , a key factor in the regulation of pluripotency and neural differentiation. *World J. Stem Cells* **6,** 305–311 (2014).
26. Chambers, I. *et al.* Nanog safeguards pluripotency and mediates germline development. *Nature* **450,** 3–8 (2007).
27. Jiang, J. *et al.* A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat. Cell Biol.* **10,** (2008).
28. Guo, G. *et al.* Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* **1069,** 1063–1069 (2009).
29. Khalfallah, O., Rouleau, M., Barbry, P., Bardoni, B. & Lalli, E. Dax-1 Knockdown in Mouse Embryonic Stem Cells Induces Loss of Pluripotency and Multilineage Differentiation. *Stem Cells* 1529–1537 (2009). doi:10.1002/stem.78
30. Sun, C. *et al.* Dax1 Binds to Oct3 / 4 and Inhibits Its Transcriptional Activity in Embryonic Stem Cells □ †. *Mol. Cell. Biol.* **29,** 4574–4583 (2009).
31. Zhang, J. *et al.* Dax1 and Nanog act in parallel to stabilize mouse embryonic stem cells and induced pluripotency. *Nat. Commun.* **5,** 1–16 (2014).
32. Cartwright, P. *et al.* LIF / STAT3 controls ES cell self-renewal and pluripotency by a Myc- dependent mechanism. *Development* (2005). doi:10.1242/dev.01670
33. Baudino, T. A. *et al.* c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes Dev.* **1,** 2530–2543 (2002).
34. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **2,** 663–676 (2006).

35. Smith, A. G., Nichols, J., Robertson, M. & Rathjen, P. D. Inhibiting Activity ( DIA / LIF ) and Mouse Development. **351,** 339–351 (1992).
36. Hirai, H., Karian, P. & Kikyo, N. Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *Biochem. J.* **438,** 11–23 (2011).
37. Matsuda, T. *et al.* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* **18,** 4261–4269 (1999).
38. Boulton, T. G., Stahl, N. & Yancopouloss, D. Ciliary Neurotrophic Factor / Leukemia Inhibitory Factor / Interleukin 6 / 0ncostatin M Family of Cytokines Induces Tyrosine Phosphorylation of a Common Set of Proteins Overlapping Those Induced by Other Cytokines and Growth Factors. *J. Biol. Chem.* 11648–11655 (1994).
39. Ihle, J. N. & Kerr, I. A. N. M. Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet.* **11,** (1995).
40. Kristensen, D. M., Kalisz, M. & Nielsen, J. H. Cytokine signalling in embryonic stem cells. *APMIS* (2005).
41. Darnell Jr, J. STATs and Gene Regulation. *Science (80-. ).* **277,** (1997).
42. Chen, X. *et al.* Integration of External Signaling Pathways with the Core Transcriptional Network in Embryonic Stem Cells. *Cell* 1106–1117 (2008). doi:10.1016/j.cell.2008.04.043
43. Kidder, B. L., Yang, J. & Palmer, S. Stat3 and c-Myc Genome-Wide Promoter Occupancy in Embryonic Stem Cells. *PLoS One* **3,** 1–14 (2008).
44. Bourillot, P.-Y. *et al.* Novel STAT3 Target Genes Exert Distinct Roles in the Inhibition of Mesoderm and Endoderm Differentiation in Cooperation with Nanog. *Stem Cells* 1760–1771 (2009). doi:10.1002/stem.110
45. Ying, Q., Nichols, J., Chambers, I. & Smith, A. BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. *Cell* **115,** 281–292 (2003).
46. Van oosten, A. L., Costa, Y., Smith, A. & Silva, J. C. R. JAK / STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nat. Commun.* (2013). doi:10.1038/ncomms1822.JAK/STAT3
47. Kingsley, D. M. The TGF-β superfamily : new members , new receptors , and new genetic tests of function in different organisms. *Genes Dev.* 133–146 (1994).
48. Derynck, R. & Feng, X. TGF- b receptor signaling. *Biochim. Biophys. Acta* (1997).
49. Heldin, C., Miyazono, K. & Dijke, P. TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390,** 465–471 (1997).
50. Fei, T. *et al.* Genome-wide mapping of SMAD target genes reveals the role of BMP signaling in embryonic stem cell fate determination. *Genome Res.* **14,** 36–44 (2010).

51. Clevers, H., Loh, K. M. & Nusse, R. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science (80-. ).* (2014). doi:10.1126/science.1248012
52. Amerongen, R. Van & Nusse, R. Towards an integrated view of Wnt signaling in development. *Development* **3214,** 3205–3214 (2009).
53. Arnold, S. J. *et al.* Brachyury is a target gene of the Wnt / b -catenin signaling pathway. *Mech. Dev.* **91,** (2000).
54. Aubert, J., Dunstan, H., Chambers, I. & Smith, A. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat. Biotechnol.* **20,** (2002).
55. Huelsken, J. *et al.* Requirement for β-Catenin in Anterior-Posterior Axis Formation in Mice. *J. Cell Biol.* **148,** 567–578 (2000).
56. Kelly, O. G., Pinson, K. I. & Skarnes, W. C. The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* 2803–2815 (2004). doi:10.1242/dev.01137
57. Liu, P. *et al.* Requirement for Wnt3 in vertebrate axis formation. *Nature* **22,** 361–365 (1999).
58. Stamos, J. L. & Weis, W. I. The b -Catenin Destruction Complex. *Cold Spring Harb. Perspect. Biol.* 1–16 (2013).
59. Valenta, T., Hausmann, G. & Basler, K. The many faces and functions of b -catenin. *EMBO J.* **31,** 2714–2736 (2012).
60. Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. & Brivanlou, A. H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* **10,** 55–63 (2004).
61. Cole, M. F., Johnstone, S. E., Newman, J. J., Kagey, M. H. & Young, R. A. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. *Genes Dev.* 746–755 (2008). doi:10.1101/gad.1642408.4
62. Kelly, K. F., Ng, D. Y., Jayakumaran, G., Wood, G. A. & Doble, B. W. β -Catenin Enhances Oct-4 Activity and Reinforces Pluripotency through a TCF-Independent Mechanism. *Cell Stem Cell* **8,** 214–227 (2011).
63. Pereira, L., Yi, F. & Merrill, B. J. Repression of Nanog Gene Transcription by Tcf3 Limits Embryonic. *Mol. Cell. Biol.* **26,** 7479–7491 (2006).
64. Wray, J., Kalkan, T., Gomez-lopez, S., Eckardt, D. & Cook, A. Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat. Cell Biol.* **13,** 838–845 (2011).
65. Wu, C. *et al.* Function of Wnt /β-catenin in counteracting Tcf3 repression through the Tcf3 – β-catenin interaction. *Development* **2129,** 2118–2129 (2012).
66. Yi, F. *et al.* Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of

embryonic stem cell self-renewal. *Nat. Cell Biol.* **13,** 762–770 (2011).

67. Marson, A. *et al.* Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell Stem Cell* **3,** 132–135 (2011).
68. Koike, M., Sakaki, S., Amano, Y. & Kurosawa, H. Characterization of Embryoid Bodies of Mouse Embryonic Stem Cells Formed under Various Culture Conditions and Estimation of Differentiation Status of Such Bodies. *J. Biosci. Bioeng.* **104,** 294–299 (2007).
69. Desbaillets, I., Ziegler, U., Groscurth, P. & Gassmann, M. Embryoid bodies: an in vitro model of mouse embryogenesis. *Exp. Physiol.* (2000).
70. Loh, K. M. & Lim, B. Perspective A Precarious Balance: Pluripotency Factors as Lineage Specifiers. *Cell Stem Cell* **8,** 363–369 (2011).
71. Kopp, J., Ormsbee, B., Desler, M. & Rizzino, A. Small Increases in the Level of Sox2 Trigger the Differentiation of Mouse Embryonic Stem Cells. *Stem Cells* 903–911 (2008). doi:10.1634/stemcells.2007-0951
72. Teo, A. K. K. *et al.* Pluripotency factors regulate definitive endoderm specification through eomesodermin. *Genes Dev.* **2,** 238–250 (2011).
73. Yu, P., Pan, G., Yu, J. & Thomson, J. A. FGF2 Sustains NANOG and Swiches the Outcome of BMP4 Induced Human Embryonic Stem Cell Differentiation. *Cell Stem Cell* **8,** 326–334 (2011).
74. Armstrong, L. Epigenetic Control of Embryonic Stem Cell Differentiation. *Stem Cell Rev. Reports* 67–77 (2012). doi:10.1007/s12015-011-9300-4
75. Bernstein, B. E. *et al.* A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* 315–326 (2006). doi:10.1016/j.cell.2006.02.041
76. Kraushaar, D. C. & Zhao, K. The Epigenomics of Embryonic Stem Cell Differentiation. *Int. J. Biol. Sci.* **9,** (2013).
77. Shim, J. & Nam, J.-W. The expression and functional roles of microRNAs in stem cell differentiation. *BMB Rep.* **49,** 3–10 (2016).
78. Marson, A. *et al.* Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **134,** 521–533 (2008).
79. Lichner, Z. & Go, E. The miR-290-295 cluster promotes pluripotency maintenance by regulating cell cycle phase distribution in mouse embryonic stem cells. *Differentiation* **81,** 11–24 (2011).
80. Card, D. A. G. *et al.* Oct4/Sox2-Regulated miR-302 Targets Cyclin D1 in Human Embryonic. *Mol. Cell. Biol.* **28,** 6426–6438 (2008).
81. Mallanna, S. K. & Rizzino, A. Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. **344,** 16–25 (2011).
82. Berardi, E., Pues, M., Thorrez, L. & Sampaolesi, M. miRNAs in ESC differentiation. *Am. Physiol. Soc.* 931–939 (2012). doi:10.1152/ajpheart.00338.2012

83. Stern, C. D. Neural induction: old problem, new findings, yet more questions. *Development* **4,** 2007–2021 (2005).
84. Levine, A. J. & Brivanlou, A. H. Proposal of a model of mammalian neural induction. *Dev. Biol.* **308,** 247–256 (2007).
85. Cai, C. & Grabel, L. Directing the Differentiation of Embryonic Stem Cells to Neural Stem Cells. *Dev. Dyn.* 3255–3266 (2007). doi:10.1002/dvdy.21306
86. Kawasaki, H. *et al.* Induction of Midbrain Dopaminergic Neurons from ES Cells by Stromal Cell – Derived Inducing Activity. *Neuron* **28,** 31–40 (2000).
87. Kim, M. *et al.* Regulation of mouse embryonic stem cell neural differentiation by retinoic acid. *Dev. Biol.* **328,** 456–471 (2009).
88. Ying, Q., Stavridis, M., Griffiths, D., Li, M. & Smith, A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* **21,** 183–186 (2003).
89. Finley, M. F. A., Devata, S. & Huettner, J. E. BMP-4 Inhibits Neural Differentiation of Murine Embryonic Stem Cells. *Dev. Neurobiol.* **4,** (1999).
90. Parisi, S. *et al.* Nodal-dependent Cripto signaling promotes cardiomyogenesis and redirects the neural fate of embryonic stem cells. *J. Cell Biol.* 303–314 (2001). doi:10.1083/jcb.200303010
91. Tropepe, V. *et al.* Direct Neural Fate Specification from Embryonic Stem Cells: A Primitive Mammalian Neural Stem Cell Stage Acquired through a Default Mechanism. *Neuron* **30,** 65–78 (2001).
92. Haegele, L. *et al.* Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression. *Mol. Cell. Neurosci.* **24,** 696–708 (2003).
93. Watanabe, K. *et al.* Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat. Neurosci.* **8,** 288–296 (2005).
94. Wilson, P. G. & Stice, S. S. Development and Differentiation of Neural Rosettes Derived From Human Embryonic Stem Cells. *Stem Cell Rev.* **0,** 67–77 (2006).
95. Elkabetz, Y., Panagiotakos, G., Shamy, G. Al, Socci, N. D. & Tabar, V. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes Dev.* 152–165 (2008). doi:10.1101/gad.1616208.1995
96. Lindsley, D. & Grell, E. Spermiogenesis without chromosomes in Drosophila melanogaster. *Genetics* (1968).
97. Buscarlet, M. & Stifani, S. The ‘ Marx ’ of Groucho on development and disease. *Trends Cell Biol.* **17,** (2007).
98. Fisher, A. L. & Caudy, M. Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* 1931–1940 (1998).
99. Paroush, Z. *et al.* Groucho Is Required for Drosophila Neurogenesis, Segmentation, and Sex Determination and Interacts Directly with Hairy-Related bHLH Proteins. *Cell* **79,** 805–815 (1994).

100. Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. & Simpson, P. Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **171,** 161–171 (1996).
101. Pinto, M. & Lobe, C. G. Products of the grg (Groucho-related Gene) Family Can Dimerize through the Amino-terminal Q Domain. *J. Biol. Chem.* **271,** 33026–33031 (1996).
102. Chen, G., Fernandez, J., Mische, S. & Courey, A. J. A functional interaction between the histone deacetylase Rpd3 and the corepressor Groucho in Drosophila development. *Genes Dev.* 2218–2230 (1999).
103. Beagle, B. & Johnson, G. V. W. AES/GRG5: More Than Just a Dominant-Negative TLE/GRG Family Member. *Dev. Dyn.* 2795–2805 (2010). doi:10.1002/dvdy.22439
104. Gasperowicz, M. & Otto, F. Mammalian Groucho Homologs: Redundancy or Specificity? *J. Cell. Biochem.* (2005). doi:10.1002/jcb.20476
105. Bajoghli, B. Evolution of the Groucho/Tle gene family: gene organization and duplication events. *Dev. Genes Evol.* 613–618 (2007). doi:10.1007/s00427-007-0167-y
106. Mallo, M., Franco, F. & Gridley, T. Cloning and developmental expression of Grg, a mouse gene related to the groucho transcript of the Drosophila Enhancer of split complex. *Mech. Dev.* **42,** 67–76 (1993).
107. Mallo, M., Gendron-maguire, M., Harbison, M. L. & Grldley, T. Protein Characterization and Targeted Disruption of Grg, a Mouse Gene Related to the groucho Transcript of the Drosophila Enhancer of split Complex. *Dev. Dyn.* **347,** 338–347 (1995).
108. Chen, G. & Courey, A. J. Groucho/TLE family proteins and transcriptional repression. *Gene* **249,** 1–16 (2000).
109. Lepourcelet, M. & Shivdasani, R. A. Characterization of a Novel Mammalian Groucho Isoform and Its Role in Transcriptional Regulation *. *J. Biol. Chem.* **277,** 47732–47740 (2002).
110. Yu, X. I. N., Li, P., Roeder, R. G. & Wang, Z. Inhibition of Androgen Receptor-Mediated Transcription by Amino-Terminal Enhancer of split. *Mol. Cell. Biol.* **21,** 4614–4625 (2001).
111. Cavallo, R. A. *et al.* Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395,** (1998).
112. Jan, Y. *et al.* A Mitochondrial Protein, Bit1, Mediates Apoptosis Regulated by Integrins and Groucho/TLE Corepressors. *Cell* **116,** 751–762 (2004).
113. Muhr, J., Andersson, E., Persson, M., Jessell, T. M. & Ericson, J. Groucho-Mediated Transcriptional Repression Establishes Progenitor Cell Pattern and Neuronal Fate in the Ventral Neural Tube. *Cell* **104,** 861–873 (2001).
114. Allen, T. *et al.* Grg1 Acts as a Lung-Specific Oncogene in a Transgenic Mouse

Model. *Cancer Res.* 1294–1302 (2006). doi:10.1158/0008-5472.CAN-05-1634
115. Jenning, S., Pham, T., Ireland, S. K., Ruoslahti, E. & Biliran, H. Bit1 in anoikis resistance and tumor metastasis. *Cancer Lett.* **333,** 147–151 (2014).
116. Zhu, C. C. *et al.* Six3-mediated auto repression and eye development requires its interaction with members of the Groucho-related family of co-repressors. *Development* **2849,** 2835–2849 (2002).
117. Wang, W. *et al.* Groucho homologue Grg5 interacts with the transcription factor Runx2 – Cbfa1 and modulates its activity during postnatal growth in mice. *Dev. Biol.* **270,** 364–381 (2004).
118. Wang, W. *et al.* Growth Defect in Grg5 Null Mice Is Associated With Reduced Ihh Signaling in Growth Plates. *Dev. Dyn.* **89,** 79–89 (2002).
119. López-ríos, J., Tessmar, K., Loosli, F., Wittbrodt, J. & Bovolenta, P. Six3 and Six6 activity is modulated by members of the groucho family. *Development* **4,** 185–195 (2003).
120. Liu, Y. *et al.* Sox2 acts as a transcriptional repressor in neural stem cells. *BMC Neurosci.* 2–11 (2014).
121. Steffen, B., Knop, M., Bergholz, U., Vakhrusheva, O. & Rode, M. AML1/ETO induces self-renewal in hematopoietic progenitor cells via the groucho related amino-terminal enhancer of split (AES) protein. *Blood* (2011). doi:10.1182/blood-2009-09-242545
122. Xia, H. *et al.* Suppression of RND3 activity by AES downregulation promotes cancer cell proliferation and invasion. *Int. J. Mol. Med.* 1081–1086 (2013). doi:10.3892/ijmm.2013.1321
123. Sonoshita, M. *et al.* Suppression of Colon Cancer Metastasis by Aes through Inhibition of Notch Signaling. *Cancer Cell* **19,** 125–137 (2011).
124. Mayshar, Y. *et al.* Identification and Classification of Chromosomal Aberrations in Human Induced Pluripotent Stem Cells. *Cell Stem Cell* **7,** 521–531 (2010).
125. Zhao, S., Nichols, J., Smith, A. G. & Li, M. SoxB transcription factors specify neuroectodermal lineage choice in ES cells. *Mol. Cell. Neurosci.* **27,** 332–342 (2004).
126. Hadjimichael, C. *et al.* Promyelotic Leukemia Protein Is An Essential Regulator of Stem Cell Pluripotency and Somatic Cell Reprogramming. *Stem Cell Reports* **8,** 1366–1378 (2017).
127. Chuang, J., Tung, L. & Lin, Y. Neural differentiation from embryonic stem cells in vitro : An overview of the signaling pathways. **7,** 437–447 (2015).
128. Costa, Â. M. S., Pereira-castro, I., Ricardo, E. & Spencer, F. GRG5 / AES Interacts with T-Cell Factor 4 ( TCF4 ) and Downregulates Wnt Signaling in Human Cells and Zebrafish Embryos. **8,** 1–20 (2013).
129. Chambers, I. & Tomlinson, S. R. The transcriptional foundation of pluripotency. *Development* **2322,** 2311–2322 (2009).
130. Sekkaï, D. *et al.* Microarray Analysis of LIF/Stat3 Transcriptional Targets in

Embryonic Stem Cells. *Stem Cells* 1634–1642 (2005). doi:10.1634/stemcells.2005-0182

131. Laing, A. F., Lowell, S. & Brickman, J. M. Gro/TLE enables embryonic stem cell differentiation by repressing pluripotent gene expression. *Dev. Biol.* **397,** 56–66 (2015).
132. Hoffman, J. A., Wu, C. & Merrill, B. J. Tcf7l1 prepares epiblast cells in the gastrulating mouse embryo for lineage specification. *Dev. Stem Cells* **1675,** 1665–1675 (2013).
133. Jho, E. *et al.* Wnt /β-Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway. *Mol. Cell. Biol.* **22,** 1172–1183 (2002).
134. Tago, K. *et al.* Inhibition of Wnt signaling by ICAT, a novel β-catenin-interacting protein. *Genes Dev.* 1741–1749 (2000).
135. Pieters, T. & Roy, F. Van. Role of cell–cell adhesion complexes in embryonic stem cell biology. *J. Cell Sci.* 2603–2613 (2014). doi:10.1242/jcs.146720