# Chapter 5

# The Role of mTOR-related Pathways in Pluripotency/Differentiation Regulation

## 5.1 Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase discovered in the 1990s as the direct target of rapamycin-FKBP12 complex in mammals [40] [341]. It is a crucial signalling node that integrates both intracellular and extracellular signals to regulate various cellular processes such as cell metabolism, growth, proliferation and survival. The mTOR protein functions in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [141]. mTORC1 comprises five components: mTOR, Raptor, mLST8, PRAS40 and Deptor [194] [196] [150] (Figure 5.1). It has been shown that Raptor facilitates the recruitment downstream targets to mTORC1 [300] [359]. PRAS40 and Deptor physically associate with mTORC1 and negatively regulate its kinase activity [319] [347]. Upon activation, mTORC1 directly phosphorylates Deptor and PRAS40, resulting in reduced physical association and full activation of mTORC1. mLST8 acts to stabilise the kinase activation loop of mTORC1, although genetic studies suggested that it may be dispensable for mTORC1 activity [461] [124]. mTORC2 is made of six components, some of them are common to mTORC1, such as mTOR, mLST8 and Deptor [124]. The mTORC2-specific subunits are mSIN1, Rictor and Protor1 [124] (Figure 5.1). Unlike the dispensable role in mTORC1, mLST8 is functionally essential for mTORC2 [124]. Rictor and mSIN1 stabilise each other and establish the structural foundation of mTORC2 [215]. Similar to its role in mTORC1, Deptor negatively regulates mTORC2 activity [319]. mTORC2 is characterised for its insensitivity to rapamycin treatment; however, it was demonstrated that prolonged rapamycin treatment abrogates mTORC2 signalling [212] [351]. Compared to mTORC1, mTORC2-related biology is less characterised.

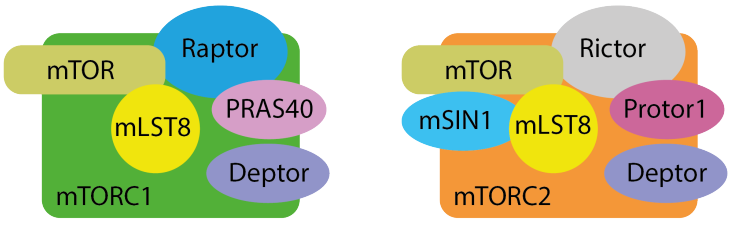


**Figure 5.1:** Structure of mTORC1 and mTORC2. mTORC1 consists of three core components: mTOR, Raptor and mLST8. It also contains two inhibitory subunits PRAS40 and Deptor. Like mTORC1, mTORC2 also contains mTOR, Deptor and mLST8. Components unique to mTORC2 are Rictor, mSin1 and Protor1.

mTORC1 is a central regulator for cell growth and metabolism. It positively regulates cell proliferation by promoting anabolic processes such as protein and lipid biosynthesis [215]. S6K1 and 4EBP1 are two of its key downstream effectors [215]. 4EBP inhibits translation through binding eIL4E to prevent the formation of eIL4F. mTORC1 phosphorylates 4EBP, which leads to its dissociation from eIL4E, allowing the cap-dependent translation to take place [44] [138]. mTORC1 also directly phosphorylates and activates S6K1, which in turn phosphorylates and activates several substrates that promotes ribosome synthesis, such as S6 and eIF4B [95] [159]. Furthermore, activated S6K promotes lipid and nucleotides synthesis via phosphorylating and activating Srebp and Cad, respectively [320] [15]. In addition to activating anabolic pathways, mTORC1 suppresses protein catabolism, such as autophagy [215]. mTORC1 was shown to phosphorylate Ulk1, which prevents it to form the autophagy driver complex with Atg13, Fip2000 and Atg101 [196]. Furthermore, a recent study demonstrated that mTORC1 regulates protein turnover by inhibiting protein ubiquitination or reducing the amount of proteasomal chaperones by inhibition of Erk5 [338].

mTORC1 integrates major metabolism related signals such as growth factors, amino acids, oxygen and energy levels to regulate downstream processes [215]. Growth factors activate mTORC1 mainly via stimulating PI(3)K-Akt pathway. Binding of insulin or other growth factors such as Igf-1 and Egf to the their cell surface receptors promotes the tyrosine kinase activity of the receptors and leads to the recruitment of Irs1 [152]. Irs1 in turn activates PI(3)K and produces PtdIns(3,4,5)P3. A key downstream effector of PtdIns(3,4,5)P3 is Akt, which is recruited to the cell membrane and activated by Pdk1 [152]. Once activated, Akt moves to the cytoplasm, where it phosphorylates and inhibits the tuberous sclerosis complex (TSC) [165] [253]. TSC is a heterotrimeric complex comprising Tsc1, Tsc2 and Tbc1d7 [87]. It functions as a GTPase-activating protein (GAP) for the small Ras-related GTPase (Rheb), which directly interacts with mTORC1 and stimulates its activity via unknown mechanism [408] [235]. Notably, S6k1, activated by mTORC1, phosphorylates Irs1 and attenuates signal transduction from receptor to PI(3)K [252]. This S6K1-dependent negative feedback loop has been shown to be deregulated in many diseases including cancer and metabolic diseases [252]. The TSC complex is also involved in response to changes in energy status and oxygen levels. Under energy deficit condition, AMPK, a major energy sensor, is activated and positively regulates Tsc2, which leads to the reduction of mTORC1 activity [166]. In response to hypoxia, Redd1 is activated and negatively regulates mTORC1 by disrupting the association of Tsc2 and 14-3-3 protein

[41] [85].

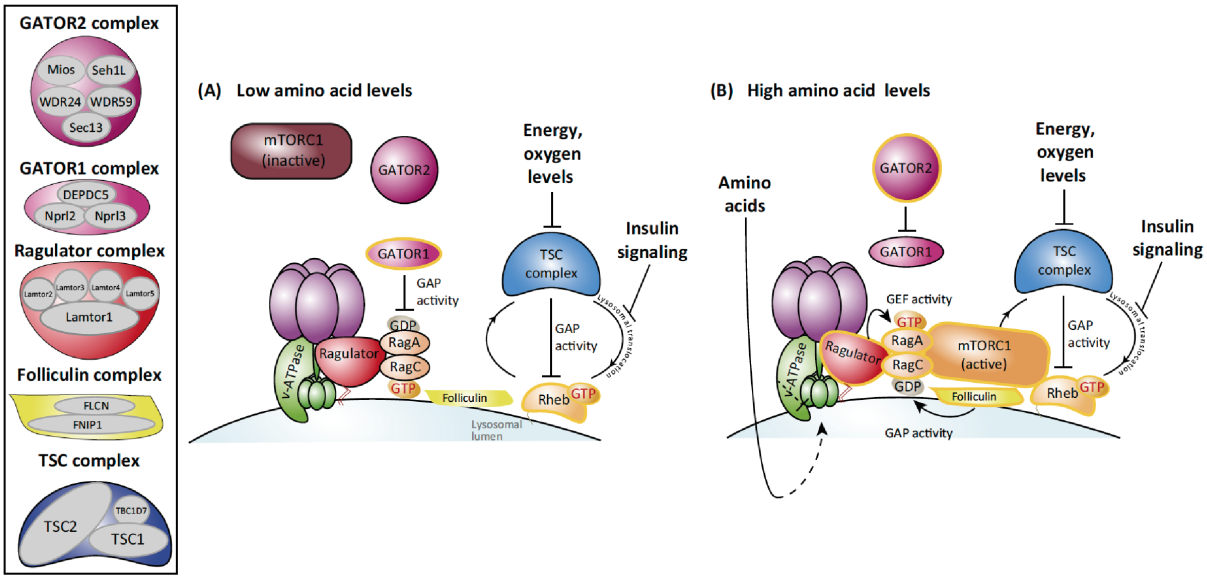


**Figure 5.2:** Structure of mTOR regulating complexes and mechanistic target of mTORC1 amino acid sensing pathway. (A) Under amino acid deprived condition, Ragulator complex and V-ATPase remain in an inhibitory state. GATOR1 complex exerts GTPase-activating protein (GAP) activity towatds RagA, keeping as inactive from recruiting mTORC1 to the lysosomal membrane. TSC complex is translocated to the lysosmal surface where it inhibits Rheb through its GAP activity. (B) Upon amino acid stimulation, Ragulator and V-ATPase undergo conformational changes. Together with folliculin, Ragulator and V-ATPase catalyse the activation of Rags complex, which recruits mTORC1 to the lysosomal membrane where it interacts with Rheb. Akt phosphorylation of TSC2 drives the TSC complex off the lysosomal surface, allowing Rheb to activate mTORC1. Image taken and adapted from Bar-Peled and Sabatini, 2014 [11].

The amount of amino acids is another strong signal that regulates the mTORC1 pathway (Figure 5.2). It was shown that the activation of mTORC1 by amino acids is independent of TSC complex, as S6K1 phosphorylation is protected from amino acid withdrawal in Tsc2-knockdown cells [299]. Amino acid sensing by mTORC1 largely relies on the heterodimeric Rag GTPases, which consist of RagA or RagB with RagC or RagD [194] [346]. The Rags complex is located on the lysosomal membrane through association with the Regulator complex, which comprises five subunits from Lamtor1 to Lamtor5 [345] [346]. Amino acid stimulation activates the Rags complex, which allows it to recruit mTORC1 to the lysosomal surface via binding of Raptor. Once recruited to the lysosomal surface, mTORC1 interacts with Rheb and becomes activated [346]. It was shown that mTORC1 senses cytosolic and lysosomal amino acids through different mechanisms. Lysosomal amino acids activate mTORC1 through transporter SLC38A9, which interacts with the Rag-Ragulator-v-ATPase complex and promotes the activation of the Rag complex [180]

[330] [432]. Cytosolic amino acids signal to mTORC1 through GATOR1 and GATOR2 complexes [10]. GATOR1 is a GAP for RagA/B and acts as an indirect inhibitor of mTORC1 [10]. GAPTOR2 positively regulates of mTORC1 via inhibiting GATOR1 [314]. Recently, a complex named KICSTOR was identified as another negative regulator that binds and recruits GATOR1 to the lysosomal membrane [448]. Sestrin2 has been identified as the primary leucine sensor for mTORC1. In the absence of leucine, Sestrin2 binds and inhibits GATOR2, which activates GATOR1, leading to mTORC1 downregulation. Another amino acid sensor is CASTOR1 (Cellular Arginine Sensor for mTORC1) [358]. Similar to Sestrin2, CASTOR1 binds and inhibits GATOR2 in the absence of arginine and dissociates upon arginine binding to enable the activation of mTORC1 [60].

mTOR deregulation is associated with a range of human diseases, such as cancer, obesity, neurodegeneration and type 2 diabetes. Although it has been shown that mTOR pathway is required in pluripotency and development, its exact function remains largely undefined. Recently, Bulut-Karslioglu et al. showed that inhibition of mTOR induces reversible pausing of mouse blastocyst development and allows prolonged culture *ex vivo*, indicating that mTOR regulates developmental timing at the peri-implantation stage [46]. Sabatini and colleagues reported that abrogation of mTORC1, by genetic loss of Rag GTPase, leads to embryonic lethality, indicating the essentiality of mTORC1 in embryonic development [101]. From a large-scale siRNA screen, Betschinger et al. found that knockdown of the mTOR regulators *Folliculin* (*Flcn*) and *Tsc2* resulted in delayed differentiation [19]. The authors also showed that Folliculin, together with its binding partners Fnip1 and Fnip2 drives differentiation by regulating the subcellular location of the transcription factor Tfe3. Genome-wide location and functional analysis showed that Tfe3 promotes the pluripotency circuitry through up-regulation of *Esrrb*.

Consistent with Betschinger's siRNA screening result, *Flcn* and *Tsc2* were identified in the CRISPR-Cas9 genome-wide screen as statistically significant genes whose loss impeded differentiation initiation. Additionally, a number of other mTORC1/2 regulators were found to be essential for the onset of differentiation, including RagA and RagC from the Rags complex, Wdr24 from GATOR2 complex and all the 5 subunits of Regulator complex. Furthermore, many of the negative regulators or components of the mTORC1/2 complexes were identified as genes whose loss accelerate mESC differentiation. This include Nprl2 and Depdc5 from GATOR1 complex, Itfg2 from KISCTOR complex, and Mlst8, which is a common component of mTORC1/2 complex. Sestrin2 and Rictor were also detected, albeit at a less significant level. One of the surprising findings from the

screen is that knockouts of mTORC1 regulators from amino acid sensing and growth factor stimulation pathways led to opposite outcomes on ESC differentiation. From the amino acid sensing aspect, mTORC1 upregulation accelerated ESC differentiation, as seen in the phenotype of GATOR1 knockout. In contrast, knockout of TSC complex, the key factor mediating the growth factor stimulation pathway, resulted in delayed differentiation phenotype. These results suggested unknown mechanisms in relation to mTORC1/2-mediated pluripotency and differentiation regulation. The following experiments in this Chapter aimed to investigate the molecular mechanisms underlying mTORC1/2-mediated pluripotency/differentiation regulation. Firstly, I verified the knockout phenotypes of GATOR1 and TSC complexes. Secondly, I compared the knockout effect of GATOR1 and TSC complexes on upstream regulators and downstream effectors of mTORC1. And finally, I analysed the transcriptomic changes of GATOR1 and TSC knockouts.

## 5.2 Results

### 5.2.1 Generation of knockout cell lines and phenotype validation

To decipher the role of mTORC1 in pluripotency and differentiation regulation, I decided to focus on the two mTORC1 inhibitory complexes: GATOR1 complex and TSC complex, whose knockouts exhibited completely different phenotypes upon differentiation from my screen. I first generated knockout ESC lines for GATOR1 components *Nprl2* and *Depdc5*, which is directly inhibited by GATOR1, as well as the TSC complex subunits *Tsc1* and *Tsc2* (Figure 5.3). Same targeting strategy was applied as discussed in Chapter 3, where *Tcf7l1* and *Apc* knockouts were described. The knockout cell lines were subsequently assessed for differentiation ability by plating in N2B27 for 27 hours followed by flow cytometry analysis. As shown in Figure 5.4 (A), phenotypes observed in each knockout cell lines agreed with screening results: *Nprl2* and *Depdc5* knockout cell lines lost Rex1GFP expression faster than parental cell line, whereas *Tsc1* and *Tsc2* knockout cell lines retained greater RexGFP positive population. For further validation, the Rex1GFPd2 cells were transduced with lentivirus expressing gRNAs targeting *Nprl2* and *Tsc2*, and plated in differentiation condition. As shown in Figure 5.4 (B), the observed single gRNA knockout phenotypes were consistent with stable knockout cell lines. To assess the differentiation progress at the cellular level, I replated cells at a low density into 2i/LIF medium after 28-hours differentiation in N2B27, and cultured them for a week before AP staining. As shown in Figure 5.4 (C), the number of AP-positive colonies was well correlated with the Rex1-GFP profiles. Thus, the screening results were confirmed with individually targeted knockout clones. Before any further investigation, it was important to make sure that the knockout phenotypes were not specific to the genetic background of E14 cells, which is the parental cell line which Rex1GFPd2 cell line was established from. To test that, I performed commitment assay using the Cas9-expressing JM8 cells transduced with gRNAs targeting *Tsc2*. As shown in Figure 5.4 (D), *Tsc2* knockout JM8 cells exhibited significantly more colonies compared to wild type, suggesting that the phenotype of delayed differentiation was not related to genetic background.

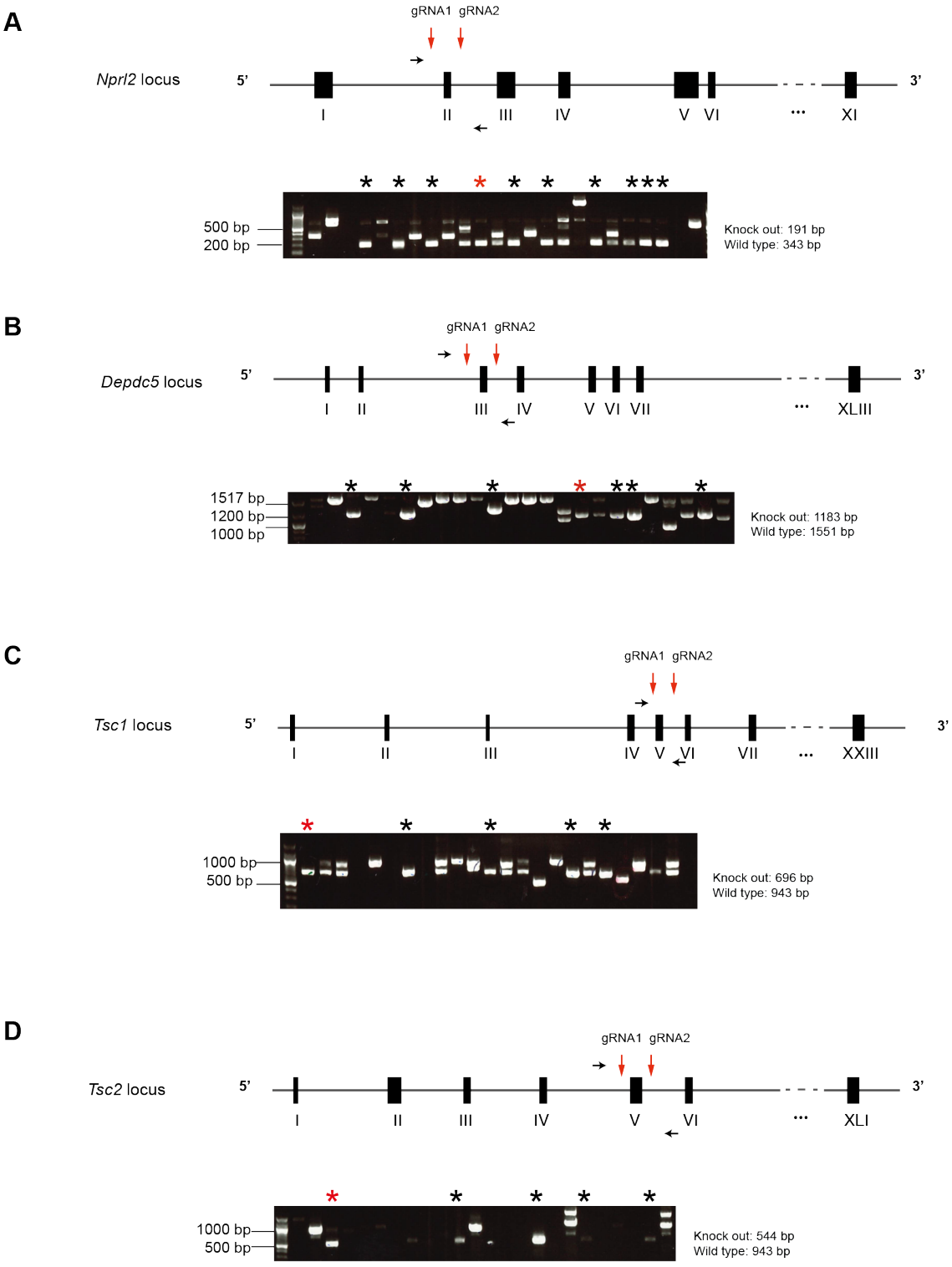


**Figure 5.3:** Generation of stable KO cell lines for mTORC1 regulating genes. (A) to (D) Schematic targeting strategy and genotyping results for *Tsc2*, *Tsc1*, *Nprl2* and *Depdc5* KO cell lines. Two gRNAs were designed to flank a critical exon and transfected into Rex1:GFPd2 cell line. A critical exon was defined as a common exon expressed in all transcript variants and when deleted, creates a frame-shift mutation. Transfected cells were plated in clonal density and single cell colonies were picked and genotyped by PCR. Black arrows indicate PCR genotyping primers. Red arrows indicate gRNA cutting sites. gRNA and PCR primer sequences are listed in Chapter 2 Table:2.1 and Table:2.2. Asterisk indicates KO clones. Red asterisk indicates KO clones used in analysis. Roman numbers indicate exon numbers.

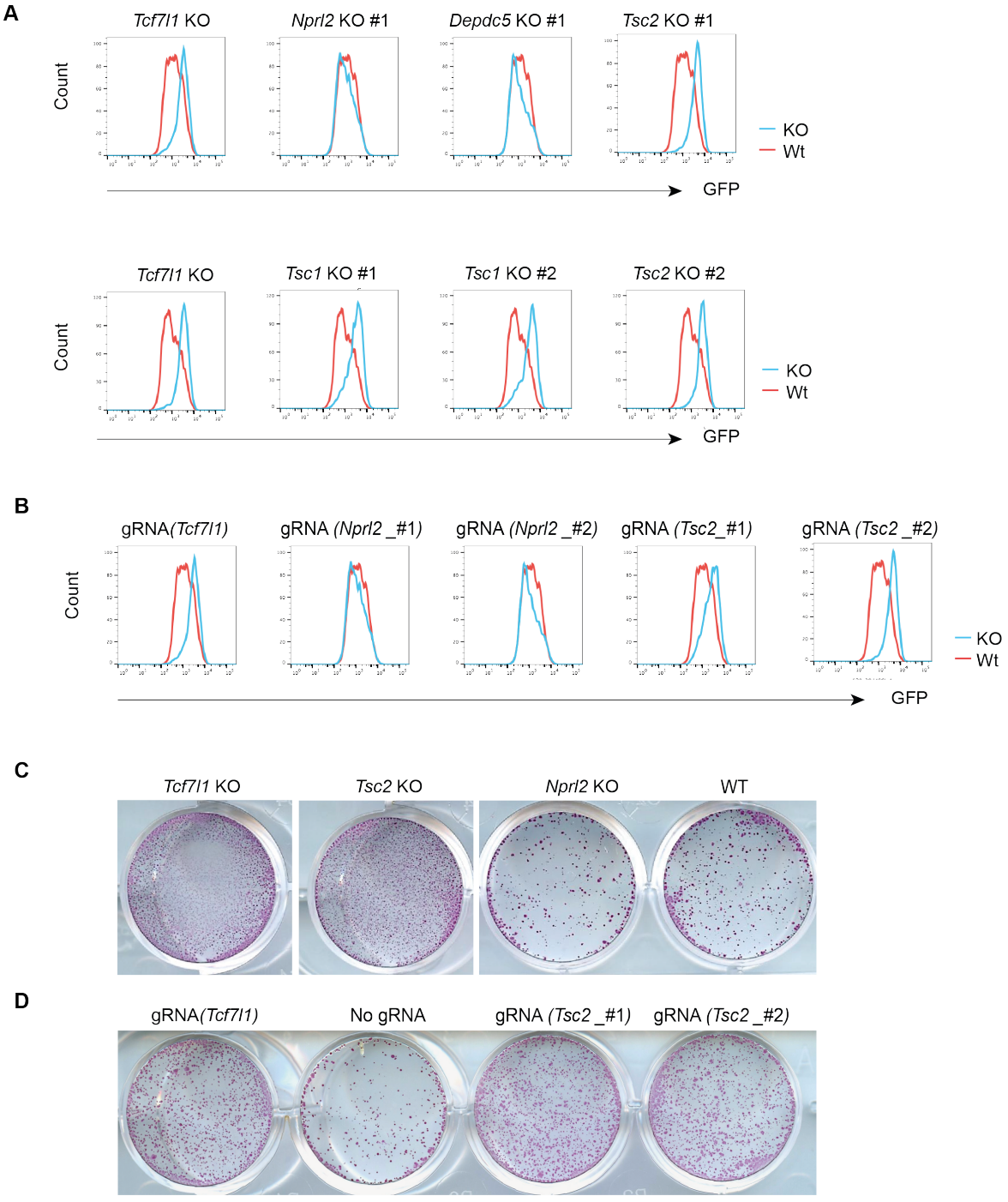


**Figure 5.4:** mTOR-related gene knockout phenotype validation. (A) Rex1GFP differentiation profile of indicated stable knockout ESC clones (knockout strategy shown in (Figure 5.1). Differentiation was induced by 2i and LIF removal. Rex1GFP was measured at 27 hours differentiation. Blue - knockout Rex1:GFP clones; Red - wt Rex1:GFP cells. (B) Rex1GFP differentiation profile of cells expressed with gRNAs targeting indicated genes. Differentiation was induced by 2i and LIF removal. Rex1GFP was measured at 27 hours differentiation. Blue -Rex1:GFP cells transduced with indicated gRNAs; Red -Rex1:GFP cells transduced with empty vector. (C) Commitment assay of stable knockout clones. Cells were kept in N2B27 medium before being replated in N2B27 supplemented with 2i/LIF. Alkaline phosphatase (AP) staining was used to visualise ESC colonies one week after replating. (D) Commitment assay using Cas9-expressing JM8 cells. Cells were transduced with Lentiviral vectors expressing indicated gRNAs.

### 5.2.2 Disruption of GATOR1 complex induces mTORC1-activated negative feedback loop

As discussed previously, mESCs cultured in serum/LIF condition exhibit heterogeneous expression of naive pluripotency markers. These two subpopulations exist in a dynamic equilibrium and can be re-established in a few passages after purifying either population by cell sorting [57]. To investigate the self-renewal ability of GATOR1 knockout cells, I sorted Rex1-GFP positive population and plated them back to serum/LIF and monitored the percentage of GFP positive population for six days. As shown in Figure 5.5 (A), wild type cells gradually lost GFP positive population for about four days until a plateau was reached at around 75%. *Tcf7l1* knockout cells hardly lost any GFP positive population: around 90% cells were expressing Rex1at day six, indicating its enhanced self-renewal ability. In contrast, both *Nprl2* and *Depdc5* knockout clones lost Rex1-GFP expression at a much faster rate than wild type cells, and plateaued at around 40% to 50% at day six, which is significantly lower than wild type cells. Strikingly, GATOR1 knockout cells struggled to retain GFP positive population even in 2i/LIF condition. They lost 5-10% of GFP-positive cells in 6 days, whilst wild type and *Tcf7l1* knockout cells retained high GFP-positive fraction. Furthermore, the Rex-GFP negative population of GATOR1-deficient cells failed to re-establish the GFP-positive naive pluripotent cells under serum/LIF condition, whereas GFP-negative wild type and *Tcf7l1* knockout cells could re-acquire Rex1 expression to approximately 30% and 50%, respectively, in four days (Figure 5.5 (B)). These observations indicate compromised self-renewal ability in GATOR1 knockout cells.

I next analysed the knockout effect of *Nprl2* and *Tsc2* on other key regulators upstream and downstream of mTORC1 by focusing on their phosphorylation status (Figure 5.5 (C)). In line with previous findings, *Nprl2* and *Tsc2* knockout cells exhibited higher phosphorylation of S6K and S6, indicating increased mTORC1 activity [165] [10]. It is known that activation of mTORC1 induces negative feedback loop through phosphorylation of Irs1 by activated S6K, which results in reduced signal transduction from receptor to Akt [252]. This is well-correlated with the phenotype of *Nprl2* knockout, where phosphorylation of Akt on S473 is considerably weaker compared to that in wild type, suggesting down-regulated Akt activity as expected. Surprisingly, *Tsc2* knockout cells exhibited strongly up-regulation of phosphorylation at S473, indicating higher Akt activity. I then moved on to analyse the downstream targets of Akt such as GSK3 and PRAS40. Consistent with Akt S473 phsophorylation, PRAS40 was highly phosphorylated in *Tsc2* knockout cells, but *Nprl2* knockout cells showed no obvious difference from wild type cells. The

phosphorylation pattern of GSK3 in each knockout cell line is well correlated with Akt phosphorylation pattern, as well as the observed differentiation phenotypes (Figure 5.5 (D)). Because GSK3 is an important node of the Wnt signalling pathway in regulating pluripotency and differentiation, I hypothesised that the opposite differentiation phenotypes of GATOR1 knockout and TSC knockout cells were caused by the difference in GSK3 regulation.

For further investigation of the GSK3 involvement in *Nprl2* knockout phenotype, *Nprl2* knockout cells were plated in '1i' condition (N2B27 with Mek inhibitor PD0325901 and LIF) supplemented with serially diluted GSK3 inhibitor CHIR99021 (Figure 5.5 (E)) (This experiment was performed by Kosuke Yusa). In the complete absence of CHIR99021, wild type cells retained around 80% Rex1-GFP positive population, whereas *Nprl2* knockout cells has only approximately 45% Rex1-GFP positive cells. Furthermore, *Nprl2* knockouts appeared to be more responsive to the changes of CHIR99021 concentration and more dependent on CHIR99021 to maintain Rex1-GFP expression, in line with the elevated Gsk3 activity indicated by western blot. Since it was speculated that the elevation of Gsk3 activity in *Nprl2* knockout cells is most likely through the negative feedback mechanism by hyperactive mTORC1 and the resulted negative feedback loop, inhibition of mTORC1 would reverse the phenotype. Indeed, as shown in Figure 5.5 (F) and (G), treating Nprl2 knockout cells with 20nM rapamycin restored the ability of pluripotency maintenance in 1i/LIF (This experiment was performed by Kosuke Yusa), and rescued the accelerated differentiation phenotype.

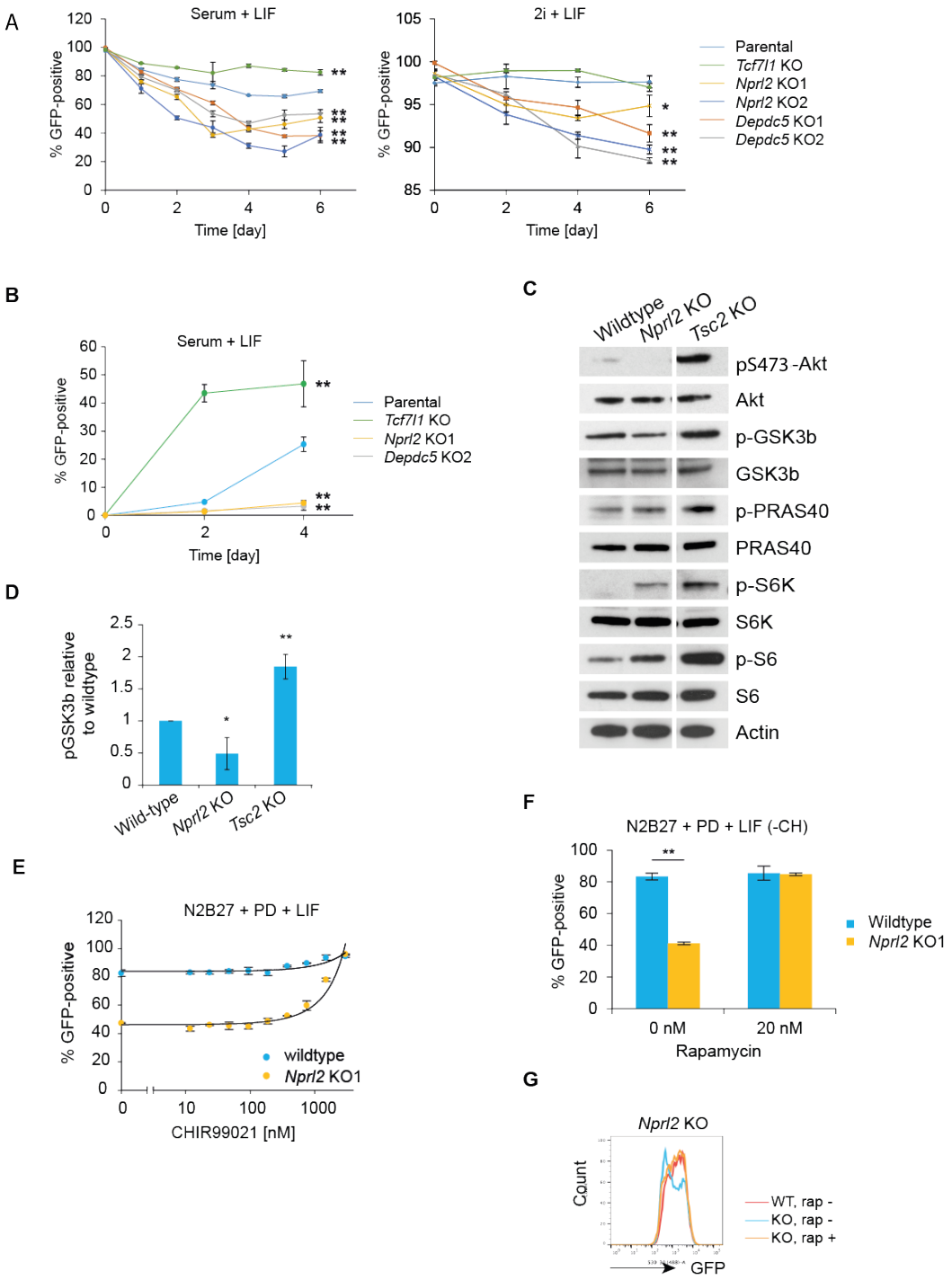


**Figure 5.5:** GATOR1 KO phenotype is resulted from activated GSK3. (A) Rex1-GFP positive population of GATOR1 key component (*Nprl2* and *Depdc5*) KO cells was sorted and maintained for six days in serum/LIF and 2i/LIF conditions. Rex1-GFP expression was monitored by flow cytometry every day. (B) Rex1-GFP negative population of *Nprl2* and *Depdc5* KO cells were plated in serum/LIF. Rex1-GFP expression was monitored by flow cytometry every day for four days. (C) WT, *Nprl2* KO and *Tsc2* KO cell lines were starved in basal medium for 2 hours and stimulated with N2B27 for 10 minutes. Cell lysate was probed with indicated antibodies. (D) Quantification of Gsk3 phosphorylation shown in (C). (E) *Nprl2* KO and WT cells were plated in N2B27+PD0325901+LIF supplemented with serially diluted GSK3 inhibitor concentration. The percentage of Rex1-GFP positive population was measured by flow cytometry. (F) Rapamycin treatment of *Nprl2* KO cells in N2B27+PD0325901+LIF. The percentage of Rex1-GFP positive population was measured by flow cytometry. (G) Rapamycin treatment rescued accelerated differentiation phenotype of *Nprl2* KO. Differentiation was induced by 2i/LIF withdrawal from N2B27. 20 nM rapamycin was added when cells were plated for differentiation. Rex1GFP expression was analysed after 27 hrs.

### 5.2.3 *Tsc2* knockout demonstrated unconventional cross-talk between mTOR and PI(3)K-Akt pathways

As mentioned in the previous section, *Tsc2* knockout mESC cells showed unconventional Akt phosphorylation pattern, namely upregulation of S473 phosphorylation (Figure 5.5 (C)). This observation suggests that, despite up-regulated mTORC1 activity, the S6K-mediated negative feedback loop does not seem to be operational: Akt activity was substantially up-regulated as shown by hyper-phosphorylated GSK3 and PRAS40 was observed. To investigate the role of hyperactivated Akt in *Tsc2* knockout mESCs, I generated *Rictor* knockout and *Tsc2**Rictor* double knockout mESC lines (Figure 5.6 (A)). Rictor is an essential component of mTORC2, which has been identified as the kinase that phosphorylates Akt at S473 and fully activates Akt. In my screen, *Rictor* was identified as a significant hit whose knockout was depleted from the Rex1-GFP population, indicating that Rictor knockout accelerates differentiation. Consistent with the screening results, *Rictor* knockout ESCs demonstrated accelerated differentiation phenotype (Figure 5.6 (B)). However, *Tsc2**Rictor* double knockout cells exhibited Rex1-GFP profile similar to *Tsc2* knockout, indicating that hyperactive Akt was not the causative factor for the delayed differentiation phenotype of *Tsc2* knockout.

To further dissect the molecular functions of mTORC2 and Akt, I analysed the phosphorylation status of the key components of Akt-mTOR pathway in *Rictor* knockout, *Tsc2* knockout and *Tsc2**Rictor* double knockout. As shown in Figure 5.6 (C), loss of mTORC2 activity did not affect the up-regulated mTORC1 activity in *Tsc2* knockout background as indicated by highly phosphorylated S6K and S6 in *Tsc2**Rictor* double knockout cells. Deletion of Rictor completely abolished Akt phosphorylation at S473 in both *Rictor* knockout and *Tsc2**Rictor* double knockout. Not much change was observed on Akt phosphorylation at T308. An Akt kinase assay was performed by Jason Yu in the lab using GSK3a as the substrate and showed that Akt activity in *Tsc2**Rictor* double knockout cells was almost abolished (Figure 5.6 (D)). However, interestingly, two Akt downstream targets PRAS40 and GSK3 retained high levels of phosphorylation in double knockout ESCs comparable to that of the *Tsc2* knockout cells, which indicates that the phosphorylation of PRAS40 and GSK3 in *Tsc2**Rictor* double knockout background is resulted from some unknown kinases other than Akt.

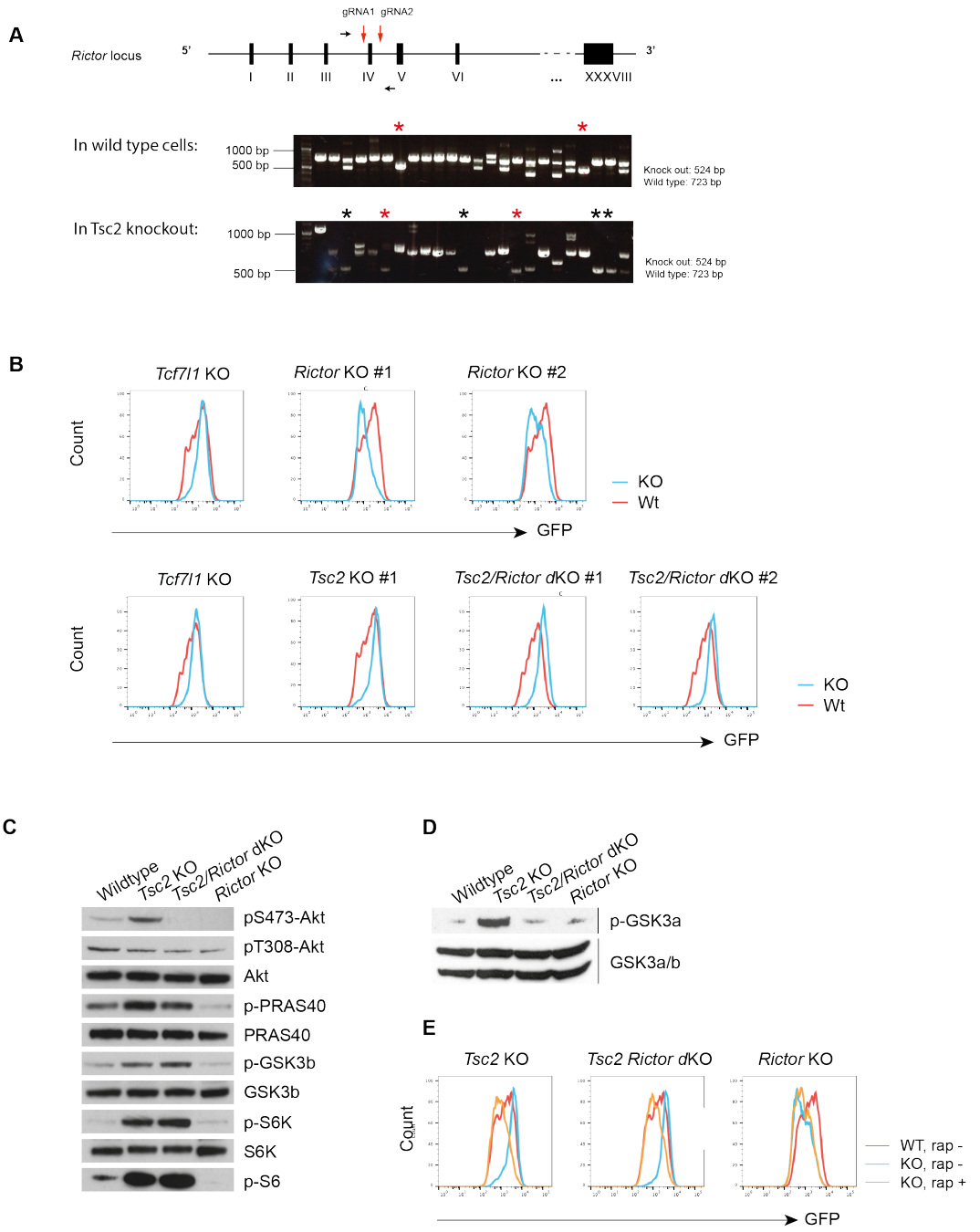


**Figure 5.6:** The unconventional cross-talk between mTOR and PI(3)K-Akt pahtways in *Tsc2* KO. (A) Schematic targeting strategy and genotyping results of *Rictor* KO. *Rictor* KO was generated using WT and *Tsc2* KO cells. Two gRNAs were designed to flank a critical exon and transfected into Rex1:GFPd2 cell line. Transfected cells were plated in clonal density and single cell colonies were picked and genotyped by PCR. Black arrows indicate PCR genotyping primers. Red arrows indicate gRNA cutting sites. gRNA and PCR primer sequences are listed in Chapter 2 Table:2.1 and Table:2.2. Asterisk indicate KO clones. Red asterisk indicates KO clones used in analysis. Roman numbers indicate exon numbers.(B) Rex1-GFP profile of *Rictor* KO, *Tsc2* KO and *Tsc2**Rictor* double KO after 27 hours differentiation in N2B27. Blue - indicated KO cells; Red - wt Rex1:GFP cells.(C) Immunoblotting result of WT, *Rictor* KO, *Tsc2* KO and *Tsc2**Rictor* double KO. Cell lysate was probed with indicated antibodies. (D) Akt kinase assay using GSK3a as a substrate (Performed by Jason Yu). (E) Rapamycin treatment rescued the impeded differentiation phenotypes of *Tsc2* KO and *Tsc2**Rictor* double KO. Differentiation was induced by 2i/LIF withdrawal from N2B27. 20 nM rapamycin was added when cells were plated for differentiation. Rex1GFP expression was analysed after 27 hrs. Red - wt rapamycin non-treated Rex1:GFP cells; Blue - indicated KO rapamycin non-treated; Yellow - indicated KO rapamycin treated.

Given that depletion of *Tsc2* strongly activates mTORC1 activity, one of the possibilities is that the kinase responsible for GSK3 phosphorylation lies downstream of the hyper-activated mTORC1. In order to test this possibility, I treated cells with rapamycin and analysed Rex1GFP profiles at 27 hours differentiation. As shown in Figure 5.6 (E), delayed differentiation phenotype in *Tsc2* knockout and *Tsc2**Rictor* double knockout ESCs was completely rescued by rapamycin treatment. And these knockout cells exhibited differentiation profiles comparable to that of the wild type cells.

### 5.2.4 Transcriptional changes in *Nprl2* and *Tsc2* knockouts

To further explore the downstream effect of *Nprl2* knockout and *Tsc2* knockout, I performed RNA-seq analysis and examined the global expression differences between these two genotypes. As these knockout cells exhibit different levels of heterogeneity of naive pluripotent cells, I purified Rex1-GFP positive populations from *Nprl2*, *Tsc2* knockout and wild type ESCs by cell sorting and performed differential gene expression analysis (Figure 5.7 (A)). At the cutoff threshold at adjusted $P$-value less than 0.1, the analysis revealed 204 up-regulated genes and 308 down-regulated genes in *Nprl2* knockout naive ESCs. *Tsc2* knockout naive ESCs exhibited substantially more differentially expressed genes: 1280 genes were up-regulated and 1390 genes were down-regulated. Surprisingly, despite the fact that only Rex1-GFP positive population was analysed, genes associated with naive pluripotency such as *Klf4*, *Tbx3*, *Esrrb* and *Tfcp2l1* were down-regulated in *Nprl2* knockout and upregulated in *Tsc2* knockout, whereas genes in relation to primed pluripotency such as *Lefty1* and *Dnmt3b* were found to be up-regulated in *Nprl2* knockout and down-regulated in *Tsc2* knockout.

To further analyse the pluripotency status of each knockout, I used curated panels of markers for general, naive and primed pluripotency[184], and examined their expression level in the RNA-Seq datasets (Figure 5.7 (B)). It was found that general pluripotency markers were expressed at a similar level across the three genotypes. Naive pluripotency markers were up-regulated in *Tsc2* knockout and primed pluripotency markers, which are associated with post-implantation epiblast, were down-regulated in *Tsc2* knockout, strongly indicating a more stabilised naive pluripotency status in *Tsc2* knockout than wild type. Complete opposite expression pattern was observed in *Nprl2* knockout, indicating that cells existed in a more primed pluripotency state despite Rex1-GFP expression. This finding indicates that although Nprl2 and Tsc2 both function as mTORC1 inhibitors, the alteration of these two genes resulted in completely different outcomes in pluripotency-

related gene expression.

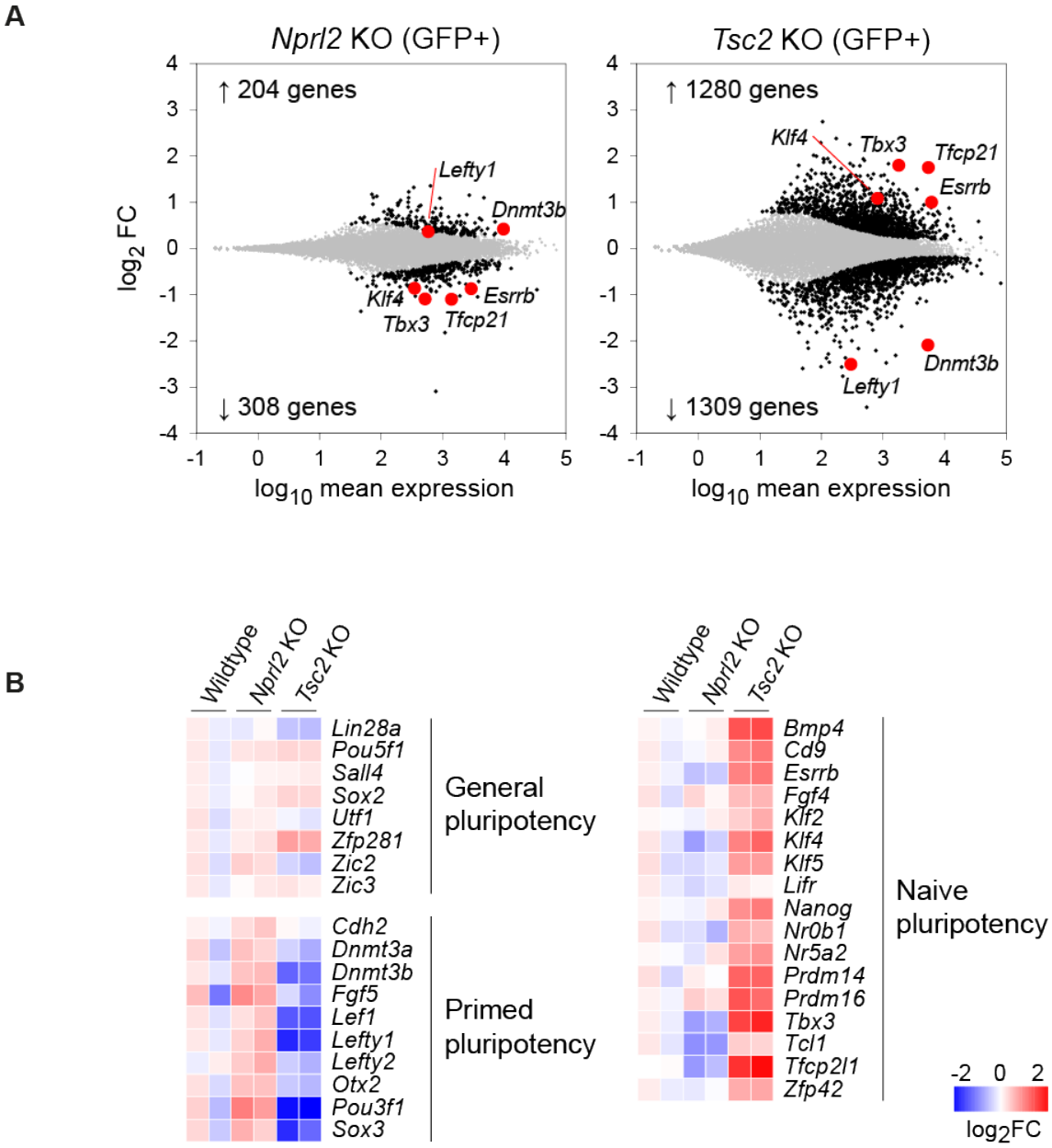


**Figure 5.7:** Transcriptional changes in *Nprl2* and *Tsc2* knockouts. (A) Global gene expression changes in Rex1-GFP positive populations of *Nprl2* and *Tsc2* knockout ESCs measured by RNA-Seq. FC: Fold change. Black dots represent differentially expressed genes. Several pluripotency/differentiation related genes were highlighted in red as examples. (B) Expression of selected general, naive and primed pluripotency markers from two independent replicates of wild type, *Nprl2* knockout and *Tsc2* knockout ESCs. Scale bar represents Log2 fold change compared to the mean TMP value of wild type replicates.

To dissect the differences in *Nprl2* and *Tsc2* knockout gene expression profiles, I divided the differentially expressed genes into four categories (Figure 5.8 (A)) and performed pathway enrichment analysis. As shown in Figure 5.8 (B), the analysis revealed that highly up-regulated genes in both genotypes were associated with metabolic processes such as amino acid metabolic process and organonitrogen metabolic process, probably as a result of stimulated mTORC1 activity. Genes that were commonly down-regulated were associated with regulation of cell cycle, notably, negative regulation of cell cycle. I postulated that constitutive active mTORC1 promotes cell proliferation thus inhibits genes that negatively regulates cell cycle. Genes that were up-regulated in *Nprl2* knockout but down-regulated in *Tsc2* knockout were associated with developmental process, which highly agrees with its accelerated differentiation phenotype. Pathways enriched from genes that were up-regulated in *Tsc2* knockout but down-regulated in *Nprl2* knockout include 'Response to oxygen containing compound', which is in line with the fact that TSC complex is involved in oxygen sensing. Interestingly, transcription-related pathway was also enriched from genes that were uniquely up-regulated in *Tsc2* knockout ESCs, which indicates that Tsc2 might be involved in transcription regulation. Whether this is through mTORC1 inhibition remain to be tested.

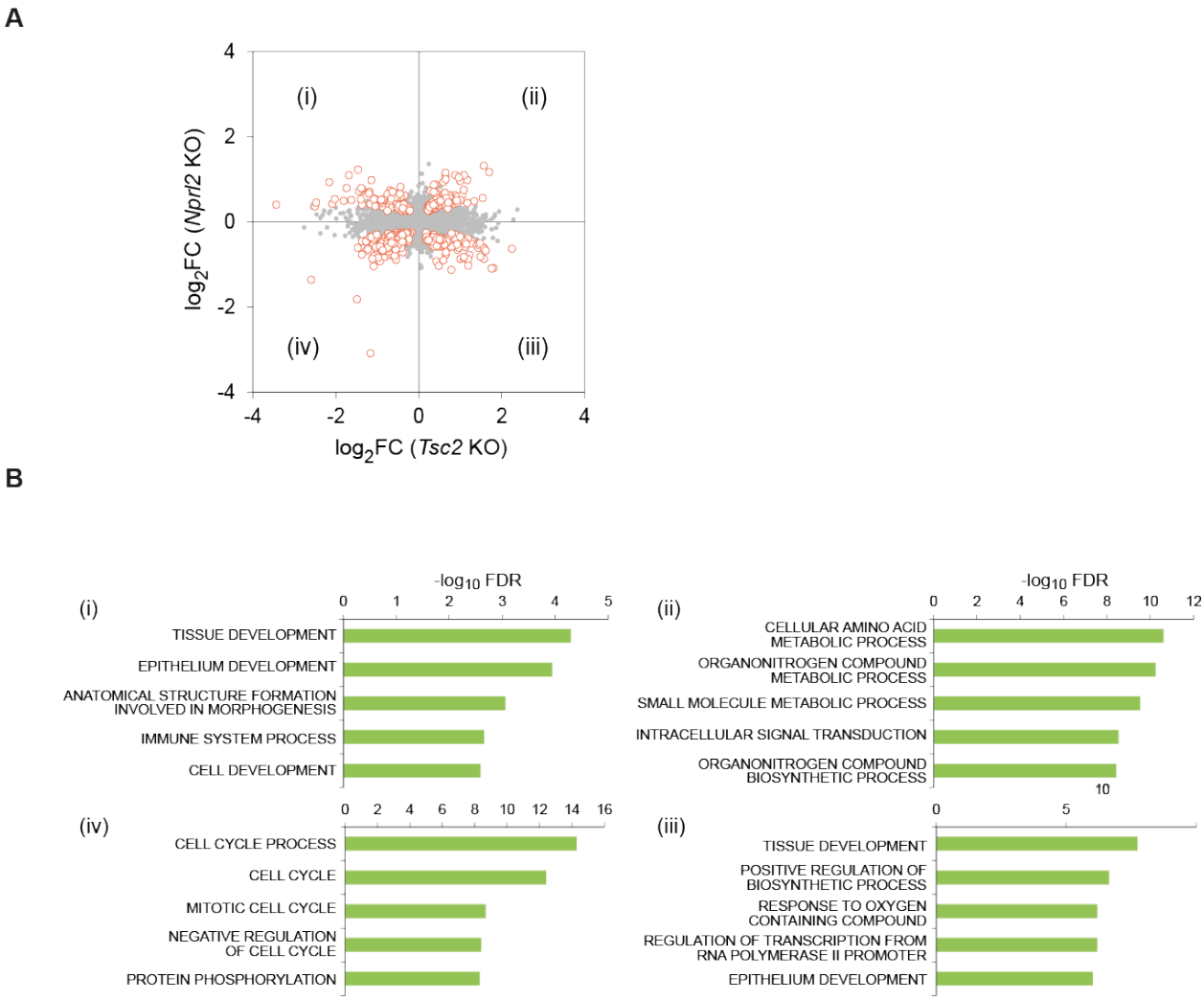


**Figure 5.8:** Pathway analysis of differentially expressed gene sets. (A) Differentially expressed genes were grouped into four categories: up-regulated in *Nprl2* knockout but down-regulated in *Tsc2* knockout (i), up-regulated in both genotypes (ii), up-regulated in *Tsc2* knockout but down-regulated in *Nprl2* knockout (iii), down-regulated in both genotypes (iv). (B) Enriched pathways of differentially expressed gene sets in each category.

## 5.3 Discussion and conclusion

The discovery of many regulators on the mTOR axis in relation to the exit from pluripotency is unprecedented, owing to the power of CRISPR-Cas9-mediated screen and the recent advance in knowledge on mTOR pathway. Although the role of mTOR and its related proteins in development has been studied previously [46] [19] [101], its exact function and the mechanism of interaction with its upstream regulators and downstream targets to make an impact on pluripotency and differentiation regulation remain a mystery. Interestingly, the screen hits covered a broad range of pathways upstream mTORC1, including growth factor stimulation, amino acid sensing and oxygen level regulation. It is noteworthy that almost all the major complexes involved in amino acid sensing were recovered and the outcome of each knockout correlated very well with its role in the pathway. Through studying the knockout phenotype of GATOR1 components, I demonstrated that the amino acid sensing-related mTORC1 regulators are involved in pluripotency regulation via influencing the Gsk3 activity by Akt (summarised in Figure 5.9).

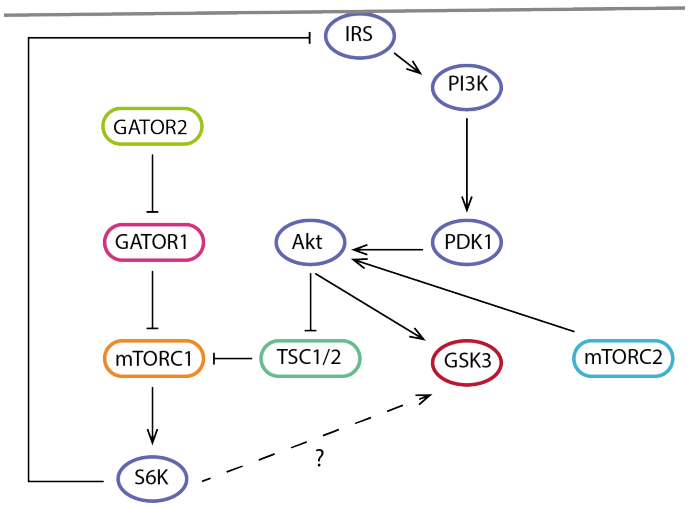


**Figure 5.9:** Summary of major relevant signalling components upstream and downstream of mTORC1 and mTORC2. mTORC1 regulation by amino acid sensing is operated via the GATOR complexes: GATOR1 negatively regulates mTORC1, whereas GATOR2 functions as a positive regulator of mTORC1 from its inhibition of GATOR1. Knocking out key components (Nprl2 and Depdc5) of GATOR1 complex confers delayed differentiation in mESCs. This is due to the increased phosphorylation of S6K resulted from mTORC1 activation, which creates a negative feedback loop to down-regulate Akt via IRS-PI3K-PDK1. The weakened Akt leads to reduced Gsk3 phosphorylation, which impairs the pluripotency status through destabilised downstream β-catenin. Knocking out TSC1/2 complex, which is another major inhibitor of mTORC1, also showed increased phosphorylation of S6K. However, conventional negative feedback loop was not operating as shown by increased Akt activity. The up-regulated Akt does not contribute to the delayed differentiation in Tsc2 knockout mESCs as knocking out Rictor (key component of mTORC2, which phosphorylates Akt and is responsible for its full activation) in Tsc2 deficient mESCs did not rescue the phenotype. There are evidence suggesting S6K rather than Akt phosphorylates and inactivates Gsk3 in MEFs [481], but wether this is the case in mESCs remains to be tested.

Gsk3 is a key negative regulator of the canonical Wnt pathway by phosphorylating and promoting degradation of β-catenin. As discussed in Chapter 1, for naive pluripotency maintenance, the major role of β-catenin is to abrogate the transcriptional repressor function of Tcf7l1. Gsk3 inhibition thus positively contributes to sustaining naive pluripotency and suppressing differentiation. Figure 5.5 (E) and (F) clearly indicates that Nprl2 knockout mESCs have an elevated Gsk3 activity and thus are more dependent on Gsk3 inhibition to maintain Rex1GFP expression. The delayed differentiation phenotype could be rescued by rapamycin (Figure 5.5 (G)), which indicates an operative negative feedback loop in Nprl2 knockout ESCs from hyperactive S6K to Akt via IRS.

One of the striking findings from the screen was that *Tsc2* knockout cells demonstrated strong delayed exit of pluripotency which is opposite of *Nprl2* knockout despite both act as mTORC1 inhibitors. This is probably also due to hyper-phosphorylated Gsk3 as shown in Figure 5.5 (C). Although there must be distinct mechanisms in the control of pluripotency regulation underlying the opposite phenotype between *Tsc2* and *Nprl2* knockout ESCs . Additionally, phosphorylation analysis revealed unexpected Akt activity in *Tsc2* knockout. Firstly, Akt appeared to be up-regulated compared to wild type cells evidenced by the hyper-phosphorylated downstream targets Pras40 and the result of Akt kinase assay Figure 5.6 (C) (D), which is contradicting the established knowledge in cancer cells that activated mTORC1 induces negative feedback loop and down-regulates Akt [252]. Furthermore, Huang et al. demonstrated that TSC complex physically interacts and activates mTORC2, which is required for the phosphorylation of Akt at S473. Mouse embryonic fibroblast cells deficient of Tsc2 lack of Akt S473 phosphorylation, which is in sharp contrast with what we observed in mouse ESCs [163] (Figure 5.6 (C)). These findings raised a possibility that the stabilised pluripotency status is resulted from upregulated Akt. However, *Tsc2\Rictor* double knockout did not rescue the phenotype observed in *Tsc2* knockout Figure 5.6 (D), suggesting that Akt is not involved in regulating Gsk3 in *Tsc2* knockout cells and that Gsk3 is rather regulated by other kinases. Given that rapamycin could rescue the phenotype caused by *Tsc2* knockout, the kinase that phosphorylates Gsk3 is most likely downstream of mTORC1. It has been described that in *Tsc2* knockout MEFs, S6K rather than Akt phosphorylates and inactivates Gsk3, which might also be the case in mouse ESCs [481]. To test this, S6k inhibitor or gRNAs targeting S6k could be used in *Tsc2* knockout ESCs.