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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Sirt1 in Trophoblast Differentiation and Placental Development

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Kanaga Sundari Rajam Arul Nambi Rajan

Committee in Charge:

Professor Mana M. Parast, Chair
Professor Mark Lawson
Professor David Natale
Professor Jianhua Shao
Professor Miles Wilkinson

2016

The Dissertation of Kanaga Sundari Rajam Arul Nambi Rajan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

In recognition of her constant love and support, I dedicate my dissertation to my family and specifically to my amma, Ezhil Arul Nambi Rajan. She has literally been by my side for every stage of my education and career, awake for every late night studying and supported me unconditionally at every step. I would not have made it this far without her and her constant love, support and motivation.

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LIST OF ABBREVIATIONS

BMP	Bone Morphogenetic Proteins
Blimp1	PR domain containing 1, with ZNF domain
c-Met	Met proto-oncogene
Cdx2	Caudal type homeobox 2
CTB	Cytotrophoblast
Ctsq	Cathespain Q
EPC	Ectoplacental cone
Eomes	Eomesodermin
Epcam	Epithelial cell adhesion molecule
Esrrb	Estrogen related receptor, beta
EVT	Extravillous trophoblast
ExE	Extraembryonic ectoderm
FGF4	Fibroblast growth factor 4
HGF	Hepatocyte growth factor
ICM	Inner cell mass
IHC	Immunohistochemistry
ISH	In situ hybridization
IUGR	Intrauterine growth restriction
Ly6e	Lymphocyte antigen 6 complex, locus E
Mash2	Achaete-scute family bHLH transcription factor 2
PCA	Principle component analysis
Prl2c2	Prolactin family 2, subfamily c, member 2
Prl3b1	Prolactin family 3, subfamily b, member 1
Prl3d1	Proactin family 3, subfamily d, member 1
Rhox4	Reproductive homeobox 4
Sirt1	Sirtuin 1
TGFβ	Transforming growth factor beta
TGC	Trophoblast giant cells
Tpbpa	Trophoblast specific protein alpha
TS	Trophoblast stem cells
Tssc3	Pleckstrin homology like domain family A, member 2
WT	Wild-type

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VITA

- 2008-2011 Undergraduate Research Assistant, University of California, Davis
- 2009-2011 Academic Peer Advisor, College of Biological Sciences, University of California, Davis
- 2011 Bachelor of Science, Genetics, University of California, Davis
- 2013-2014 Teaching Assistant, Salk Institute Mobile Science Lab
- 2016 Doctor of Philosophy, Biomedical Sciences, University of California, San Diego

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Sirt1 in Trophoblast Differentiation and Placental Development

by

Kanaga Sundari Rajam Arul Nambi Rajan

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Mana M. Parast, Chair

The placenta is a fetal-derived transient organ critical for pregnancy and fetal development. During gestation, this organ is responsible for implantation into the maternal uterus as well as facilitating gas and nutrient exchange between the mother and fetus. Defects in placental development result in pregnancy complications such as preeclampsia and intrauterine growth restriction (IUGR). The damage done by these defects can last beyond pregnancy. Not only will placental defects compromise

the health of mother and fetus during gestation but will also predispose infants to adult-onset diseases such as diabetes, obesity, and cardiovascular disease.

Sirtuin1 (SIRT1) is a NAD-dependent protein deacetylase expressed in an array of tissues and implicated in many different disease models. It regulates a variety of cellular processes such as apoptosis, inflammation, and differentiation, through deacetylation of specific proteins. Sirt1 is considered a key metabolic sensor and is therefore often implicated in metabolic diseases such as diabetes and obesity. Homozygous *Sirt1*-null mice have previously been reported to be growth restricted. However, though Sirt1 has been implicated in pregnancy, it has never been studied specifically in placental development and trophoblast differentiation.

For my dissertation, I explored the role of Sirt1 in mouse trophoblast differentiation and placental development. Using Sirt1-null mice, I found that in the absence of Sirt1, embryos die at e13.5 and placentas are consistently smaller. Upon investigation of the null placentas, I observed defects in both the labyrinthine layer and the junctional zone. Differentiation of wild-type and Sirt1-null trophoblast stem (TS) cells showed that, in the absence of Sirt1, TS cells fail to terminally differentiate and instead are halted in an Epcam⁺ labyrinthine trophoblast progenitor state. This defect is due, at least in part, to dysregulation of c-Met signaling. These findings elucidate a new role for Sirt1 during trophoblast differentiation, and indicate a potential pathway through which abnormal placental development can contribute to fetal growth restriction.

Chapter 1

Introduction

1.1 Placenta Development

The placenta develops from the trophectoderm (TE), the outer layer of the blastocyst. Due to ethical and technical difficulties of studying the human placenta, mouse models are instrumental in studying placental development. In mouse, this layer is divided into the polar and mural TE (Watson and Cross, 2005). The polar trophectoderm, consisting of cells adjacent to the inner cell mass (ICM), is the source of mouse trophoblast stem (TS) cells, which have the potential to differentiate into all of the terminal trophoblast lineages. These cells are maintained in a proliferative state through fibroblast growth factor (FGF) signaling. FGF4 signaling from the ICM interacts with the FGFR2 in the polar trophectoderm to propagate the population of undifferentiated TS cells (Tanaka et al., 1998). Meanwhile, the rest of the TE that is not immediately in contact with the ICM, the mural TE, give rise to the first wave of differentiated, invasive primary trophoblast giant cells that facilitate implantation into the maternal uterus at e4.5.

Immediately post-implantation, the polar trophectoderm expands into the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC) (Gardner et al., 1973; Cross et al., 2003). Subsequently, the chorion develops from the ExE, while portions of the inner cell mass develop into extraembryonic mesoderm, giving rise to the allantois (Rossant and Cross, 2001). These two layers come together at e8.5, initiating chorioallantoic attachment. This is the start of placental branching morphogenesis through folding of the chorion to develop the labyrinthine layer. *Gcm1* expression at specific clusters in the chorionic plate appear to initiate branching, with *Gcm1* expression maintained at the distal tip of outgrowths for as long as the branching continues (Anson-Cartwright et al., 2000; Watson et al., 2011). Cells in the

chorion differentiate further into three trophoblast subtypes that make up the labyrinth. This layer of the placenta facilitates gas and nutrient exchange between the mother and fetus.

Meanwhile, the mouse EPC, marked by expression of *Tpbpa*, develops into the spongiotrophoblast layer of the placenta (Carney et al., 1993). The spongiotrophoblasts support the labyrinthine layer and give rise to both invasive glycogen trophoblasts and various subtypes of trophoblast giant cells (TGC). Glycogen trophoblasts are poorly characterized, but, as their name suggests, store glycogen for growth of the embryo in late gestation. Spongiotrophoblasts are precursors to secondary trophoblast giant cells, which are hormone-secreting trophoblasts. Spiral-artery associated TGC invade the maternal decidua and remodel maternal arteries (Simmons et al., 2007).

Human TS cells have not yet been identified; however, the closest, most similar cell population to mouse TS cells is cytotrophoblasts (CTB). They give rise to two main human trophoblast lineages, the multinucleated syncytiotrophoblast (STB) in chorionic villi and invasive extravillous trophoblast (EVT) at the implantation site. The human chorionic villi regulate nutrient and gas exchange between maternal and fetal blood, like the mouse labyrinth. Similar to TGC, human EVT invade the maternal uterus during implantation and remodel maternal vessels (Figure 1.1).

1.2 Mouse Trophoblast Differentiation

One of the strengths of the mouse model is the ability to derive mouse trophoblast stem cells, which can be maintained and manipulated, *in vitro* and accurately recapitulate *in vivo* cell types (Figure 1.2). Mouse TS cells can be isolated from outgrowths of e3.5 mouse blastocysts (Oda et al., 2006). These cells can be

Mouse	Human
Trophoblast Stem (TS) Cells	Cytotrophoblasts (CTB)
Syncytiotrophoblast (SynT-I and SynT-II)	Syncytiotrophoblast (STB)
Spongiotrophoblast	Cell Column Trophoblast
Trophoblast Giant Cells (P-TGC, C-TGC, SpA-TGC)	Extravillous Trophoblast

Figure 1.1 Trophoblast subtypes in mouse and human placentas

A list of trophoblasts in mouse and the comparable trophoblasts known in humans thus far.

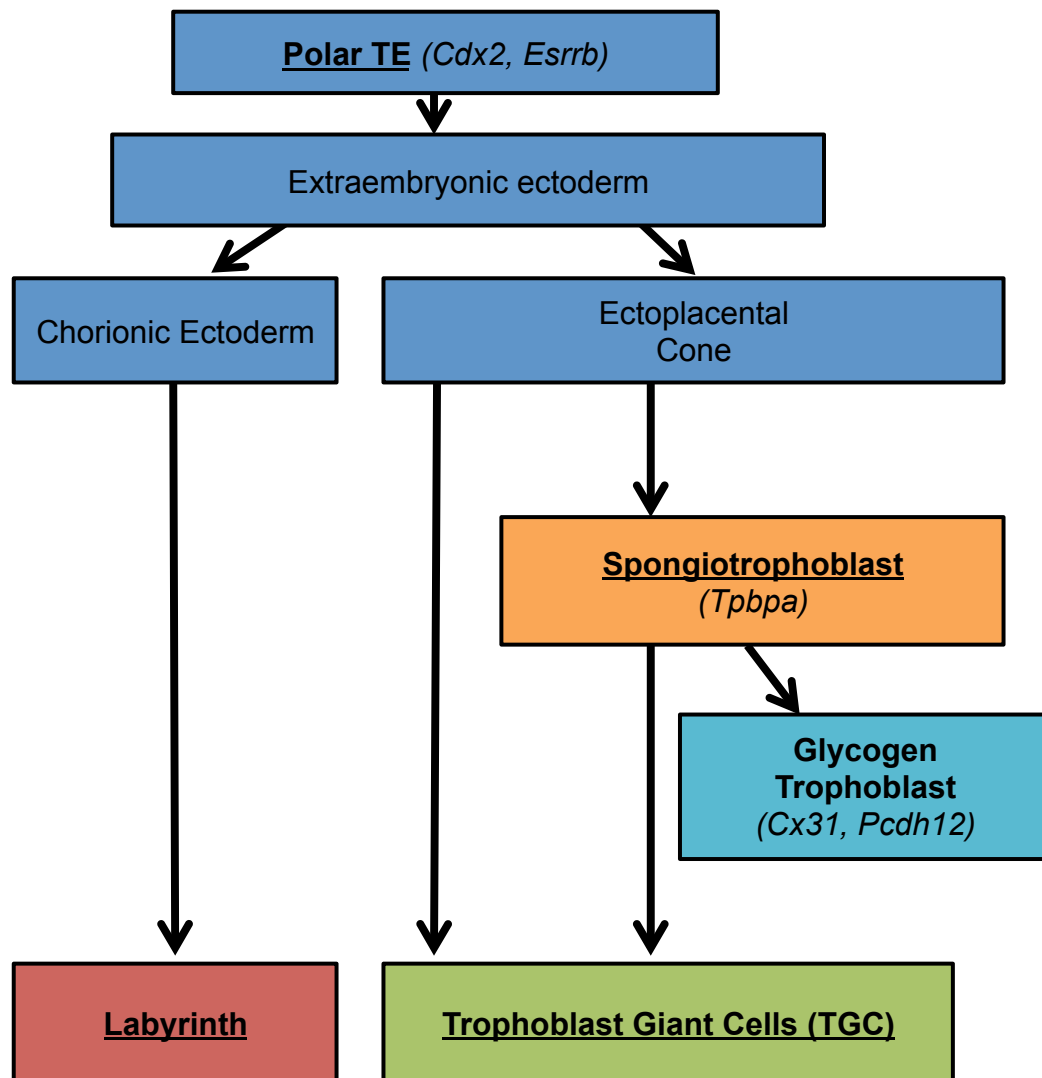


Figure 1.2 Trophoblast stem cell differentiation

Both in vivo and in vitro, trophoblast stem cells give rise to all of the known trophoblast lineages. This includes labyrinthine, spongiotrophoblast, glycogen cells and TGC

maintained in an undifferentiated, proliferative state in the presence of Activin A, FGF4, and heparin (Tanaka et al., 1998; Erlebacher et al., 2004). Differentiation can be initiated by removal of these three factors. The different trophoblast lineages can then be identified by expression patterns of specific markers. Undifferentiated TS cells express the genes *Cdx2* (Strumpf et al., 2005), *Eomes* (Russ et al., 2000) and *Esrrb* (Pettersson et al., 1996), genes that are necessary for the establishment and maintenance of TS cells. These markers decrease with trophoblast differentiation.

Spongiotrophoblasts are *Tpbpa*⁺ and are precursors for invasive trophoblast lineages (Carney et al., 1993). One of the latter lineages is the glycogen cell (GC) (Coan et al., 2006). These cells express Connexin 31 (Cx31) (Coan et al., 2006) and Protocadherin 12 (PCDH12) (Bouillot et al., 2006). Together, the spongiotrophoblast and glycogen cells form the junctional zone of the mouse placenta. The main role of the junctional zone, and specifically the spongiotrophoblast, is to support and give rise to TGCs. Trophoblast giant cells (TGC) are a diverse group of trophoblasts that all undergo endoreduplication, creating large polyploidy cells with bi- and multi-lobed nuclei (Simmons et al., 2007). There are four known subtypes of mouse TGC (Simmons et al., 2007; Hu and Cross, 2010). Each group has a different function, arises at different times during gestation, and is aptly named based on the location and function within the placenta (Figure 1.3).

Parietal TGCs (P-TGCs) originate from the mural trophoblast as the first form of differentiated trophoblasts (primary TGC) that line the implantation site, forming a barrier between the EPC and maternal decidua. These cells are identified by expression placental lactogen 1 (*Prl3d1*), placental lactogen 2 (*Prl3b1*) and proliferin (*Prl2c2*). A second wave of P-TGC (secondary TGC) then differentiates from

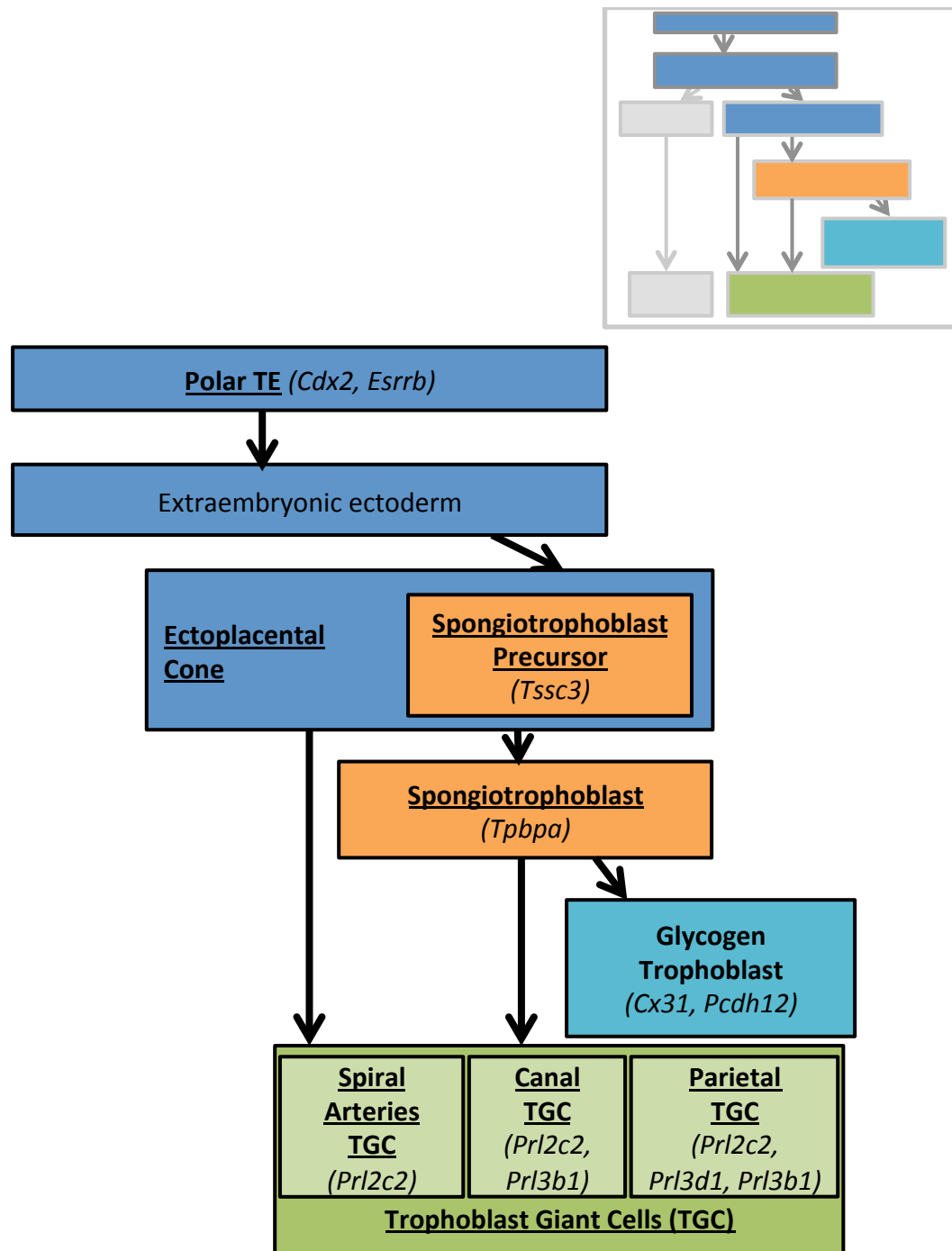


Figure 1.3 Trophoblast differentiation into invasive trophoblast lineages

The ectoplacental cone gives rise to several invasive trophoblast lineages. The spongiotrophoblasts give rise to both glycogen trophoblasts and trophoblast giant cells.

the *Tpbpa*⁺ precursors in the EPC later in gestation. Spiral artery TGC (SpA-TGC), characterized by expression of *Prl2c2*, line the maternal spiral arteries to facilitate maternal blood flow. Similarly, canal TGC (C-TGC) line the canals that bring maternal blood to the placenta. These cells are recognized by their expression of *Prl2c2* and *Prl3b1*. Despite their differences, all of these TGC are large, invasive, hormone secreting trophoblasts.

Finally, the placental labyrinth consists of three different trophoblast lineages: sinusoidal TGC (S-TGC) and two syncytiotrophoblast layers, SynT-I and SynT-II (Figure 1.4) (Simmons et al., 2008). These cells separate the maternal sinusoids from the fetal capillaries. Even prior to chorioallantoic fusion, the chorion is pre-patterned with 3 different layers. At the apical surface of the chorion are cells that will give rise to a fourth category of TGC, the S-TGC. These cells are open to maternal blood and will surround the maternal sinusoids. S-TGCs are identified by expression of *Prl3b1* and cathepsin (*Ctsq*). Cells below this layer express the gene *SynA* and give rise to SynT-I. The cells at the base of the chorion express the markers *SynB* as well as *Gcm1* (Anson-Cartwright et al., 2000; Simmons et al., 2008; Watson et al., 2011). This layer ultimately gives rise to SynT-II, which is closest to the fetal endothelial cells. The trilineage labyrinth regulates appropriate gas, nutrient and waste exchange between the mother and fetus.

Recent studies have identified several progenitor cell populations within the mouse placenta. *Tssc3* was identified as a marker of a spongiotrophoblast precursor and null mice exhibit defects in spongiotrophoblast *in vivo* (Frank et al., 2002). Further investigation demonstrated that *Tssc3/Phlda2* interacts with the PI3K/AKT pathway to regulate *Mash2/Ascl2*, a previously established spongiotrophoblast

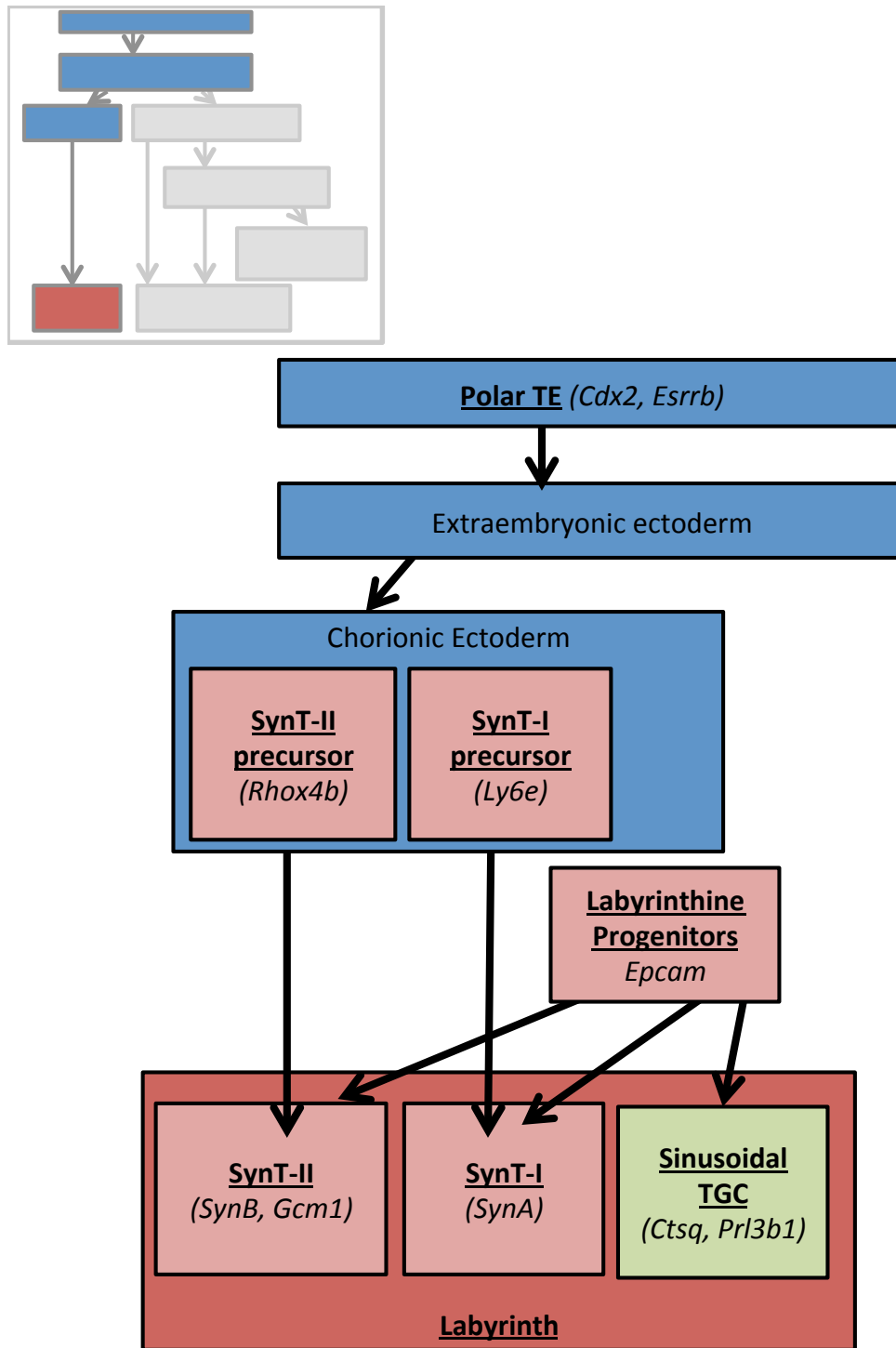


Figure 1.4 Trophoblast differentiation into labyrinthine lineages

Mouse TS cells can give rise to all of the three labyrinth trophoblast lineages: syncytiotrophoblast layer I and II as well as sinusoidal TGC. There are a few known progenitor populations of cells that can give rise to each of these lineages.

progenitor marker (Takao et al., 2012). Blimp1/Prdm1 positive cells, meanwhile, are progenitor cells that give rise to several invasive trophoblast lineages, including mature SpA-TGCs, C-TGCs and glycogen trophoblasts (Mould et al., 2012). A few labyrinthine progenitor cell markers have also been recently identified. Ly6e is a general labyrinthine progenitor marker, which is not well understood, but cells expressing this marker may preferentially develop into SynT-I (Hughes et al., 2013). Meanwhile, Rhox4-positive cells may represent precursors of SynT-II (Watson et al., 2011). More recently, Epcam has been identified as a multipotent labyrinthine progenitor marker. These cells specifically express high levels of Epcam, give rise to all three labyrinthine trophoblast sublineages (Ueno et al., 2013), and are dependent on cMet/HGF signaling for maintenance. Most of these progenitor cell types have recently been identified, and little is known about the upstream signaling pathways that regulate their self-renewal versus differentiation.

1.3 Sirtuin1 (Sirt1)

Sirtuin1 (Sirt1) is an NAD⁺ dependent protein deacetylase that is part of the Silent Information Regulator (SIRT or Sirtuin) family of proteins. All of these proteins are made up of NAD-dependent protein deacetylases and adenosine diphosphate (ADP)-ribosylases. There are seven known isoforms (Sirt1-7) and each have different cellular locations, substrates and functions.

Sirt1 is the mammalian homolog of yeast Sir2, which was the original Sirtuin that was discovered in yeast as a histone deacetylase responsible for gene silencing and increasing yeast lifespan (Kaeberlein et al., 1999; Imai et al., 2000). Since then, the field has grown to identify many non-histone substrates of mammalian Sirt1. This wide array of substrates include PPAR γ , FoxO1, HNF4a, and p53, among others

(Leibiger and Berggren, 2006). With so many different substrates, Sirt1 is implicated in many cellular processes, including, but not limited to, apoptosis (Vaziri et al., 2001), cellular cytoprotection (Chen et al., 2009), inflammation (Wu et al., 2012) and differentiation (Hisahara et al., 2008; Shakibaei et al., 2012; Simic et al., 2013b). Given that mammalian Sirt1 regulates many transcription factors, it most commonly localizes to the nucleus; nevertheless, some studies suggest it is capable of shuttling to the cytoplasm (Tanno et al., 2007). Sirt1 is fairly ubiquitously expressed in mammalian tissues including the brain (Hisahara et al., 2008), heart (Alcendor et al., 2007; Chen et al., 2009) and metabolic tissues such as adipose (Picard et al., 2004; Qiang et al., 2012).

To investigate Sirt1 *in vivo*, several groups have derived total Sirt1 knockout mouse lines on different genetic backgrounds. Interestingly, each of these lines exhibits different developmental defects and lethality. On the 129/Sv background, homozygous Sirt1-null mice are postnatal lethal and have defects such as pulmonary infection and atrophy of exocrine pancreatic epithelium (McBurney et al., 2003). Another group created null mice on a mixed background of C57BL/6 and 129/Sv and again observed postnatal lethality along with defects such as reduced sperm, cardiac defects and exencephaly (Cheng et al., 2003). However, mice on the mixed backgrounds of either 129SvEV/FVB or 129SvEv/FVB/BlackSwiss exhibited embryonic lethality (Wang et al., 2008). Despite the variability in lethality and developmental defects, all of these Sirt1-null mouse models are growth restricted (Cheng et al., 2003; McBurney et al., 2003; Wang et al., 2008). Though some of the tissues of these null mice have been studied, the placenta and gestational tissues of Sirt1-null mice have yet to be investigated.

However, Sirt1 has been studied in human pregnancy. Specifically, few studies have implicated a role for placental Sirt1 during labor. Reduced placental Sirt1 expression after labor in conjunction with anti-inflammatory responses in placental explants treated with Resveratrol, a Sirt1 activator, suggest that placental Sirt1 may regulate inflammation and inflammation-induced onset of labor (Lappas et al., 2011; Tsai et al., 2015). Sirt1 may also play a cytoprotective role in the placenta against oxidative stress. Placental Sirt1 may regulate the anti-apoptotic factor NDRG1 through the Sirt1-p53 axis in response to hypoxia (Chen et al., 2006). Sirt1 may also protect the placenta from oxidative stress and insulin resistance caused in disease states, such as diabetes and preeclampsia. Activation of placental Sirt1 facilitated glucose transport, through expression of Glut1 (Lappas et al., 2012). Beyond protecting trophoblast, placental Sirt1 could also contribute to the high adiposity observed in offspring resulting from maternal high fat diets during gestation, as Sirt1 was found to regulate fatty acid transport by regulating lipoprotein lipase (LPL) in the placenta (Qiao et al., 2015). Based on studies in human JEG3 cells, Sirt1 suppresses LPL through PPAR γ (Qiao et al., 2015). Aside from these few studies, the role of Sirt1 in trophoblast differentiation and placental development is still poorly understood.

Chapter 2

Materials and Methods

2.1 Mouse trophoblast stem cells

Sirt1^{+/+} (wild-type), Sirt1^{-/-} (Sirt1-null), 432e (wild-type #2), 29B (Sirt1-null #2), 55D (wild-type #3), 55C (Sirt1-mutant #1) and A42B (Sirt1-mutant #2) were derived from e3.5 blastocysts of mice on the 129/Sv background as previously described (Oda et al., 2006). Briefly, blastocysts were isolated and plated on murine embryonic feeder (MEF) cells. The resulting lines were adapted to feeder-free conditions and maintained in mouse TS growth media consisting of RPMI 1640 cell culture medium (Corning), 20% fetal bovine serum (Gibco), 1mM sodium pyruvate (Invitrogen), 100uM β -mercaptoethanol (Invitrogen), 2mM L-glutamine (Corning) and supplemented with 25 μ g/ml fibroblast growth factor (FGF4; Sigma), 10 μ g/ml Activin A (Stemgent) and 1 μ g/ml Heparin (Sigma). To induce trophoblast differentiation, FGF4, Activin A and Heparin were removed. Differentiation timecourses were performed for 7 days, in triplicate, and collected for RNA or protein. Serum-free media consisted of RPMI, 1mM sodium pyruvate, 100uM β -mercaptoethanol, 2mM L-glutamine and cells were usually serum starved for 5 hours prior to treatment (Tanaka et al., 1998).

Sirt1 knockdown experiments were performed using pLKO.1-based shRNA constructs (Sigma). 5 constructs were initially tested but both a single knockdown and combination were ultimately used because they produced maximum Sirt1 knockdown. To make the knockdown lines, TS cells were transfected overnight with 4 μ g DNA, 10 μ l Lipofectamine (Invitrogen) and 250 μ l OptiMEM (Gibco) in complete TS growth media. The following day, cells were switched to fresh growth media for one rest day and then treated with puromycin for selection. Knockdown was evaluated by western blot.

Trophoblast invasion *in vitro* was determined by Transwell Invasion assays (Parast et al., 2009). Transwell membranes (8.0- μ m pore inserts in 24-well BioCoat Chambers; BD Biosciences) were coated with 100 μ l cold Matrigel (BD Bioscience) after diluting it 1:20 in cold RPMI 1640. Gels were left to solidify at 37°C for 2 hours. Cells were differentiated for 7 days and 10^5 cells were re-plated on top of the Matrigel and left to invade for 3 days. To induce invasion, fresh media was added to the bottom chamber each day. On the third day, cells were fixed in 4%PFA/PBS for 10 minutes and then washed with PBS three times. Remaining cells on the top of the membrane were scraped off, leaving invasive cells on the bottom of the membrane. These cells were permeablized with 0.5% TritonX-100 for 10 minutes, washed with PBS three times and then stained with DAPI and Phalloidin. Cells were imaged and counted.

Several small molecule treatments were performed on WT and Sirt1-null TS cells. To determine if the undifferentiated null TS cell phenotype could be rescued with BMP signaling, cells were treated and passaged three times in growth media supplemented with 100ng/ml BMP4 (StemRD). Dosage treatment from 0 to 100 ng/ml HGF (Perotech; 315-23) was used to identify concentration of maximum c-Met signaling. Cells were first serum starved and then pulsed with HGF for 15minutes prior to collection. Sirt1 inhibitors were used at 50uM for Sirtinol (Calbiochem) and at 100uM for Ex527 (Sigma E7034) in standard differentiation media. Resveratrol (Cayman Chemical) was used at 25uM in standard growth or differentiation media. Cells treated with ActivinA to evaluate Activin signaling were also serum starved for 5 hours prior to treatment.

2.2 Animals

All mouse work was approved by the UC San Diego Animal Care and Use Committee. Sirt1-null and Sirt1-mutant mice on the 129/Sv background were received from Dr. Michael McBurney (Ottawa Health Research Institute, Ottawa)(McBurney et al., 2003). We also obtained Cyp19-Cre on the B6 background, originating in Dr. Gustavo Leone's lab, from Dr. Sarkis Mazmanian (California Institute of Technology)(Wenzel and Leone, 2007). Sirt1-floxed mice (Li et al., 2007) were purchased from Jackson Laboratory (B6;129-*Sirt1*^{tm1Ygu}/J; stock 008041). All three mouse lines were previously derived and characterized (McBurney et al., 2003; Li et al., 2007; Wenzel and Leone, 2007).

Timed matings were performed to identify embryonic timepoints. Mice were set up in the afternoon and checked the following morning for copulation plugs (e0.5). Mice were sacrificed at e3.5 for mouse TS cell line derivation. Pups were dissected at e13.5 and e16.5 for embryo and placental evaluations. Embryos and placentas were weighed, then fixed in 10% neutral-buffered formalin and embedded in paraffin for sectioning. For the Cre-lox recombination experiments, both embryonic and placental tissues were collected, weighed and genotyped. Previously published primers (McBurney et al., 2003; Li et al., 2007; Wenzel and Leone, 2007) were used to genotype samples from all of these mice. Primer sequences are shown in Table 1.

2.3 Trophectoderm specific knockdown

Previous studies show that trophectoderm-specific knockdowns can be performed using lentiviral infection of intact blastocysts (Okada et al., 2007; Kaufman et al., 2014). Lentivirus was produced for both a CMV-GFP vector control as well as a Scramble and Sirt shRNA. For the Sirt1 shRNA, a combination of 3 shRNA constructs (construct #3, #4, #5) was used to maximize knockdown. For lentiviral packaging,

HEK293FT cells (Life Tech) were cultured based on standard (Life Tech) protocol and transfected with 6.5µg of each expression or shRNA construct and proportional amounts of Delta 8.2 and VSV-G using Lipofectamine 2000. After an overnight transfection, 7mls of fresh media was added. Virus was then collected 48 and 72 hours after transfection and concentrated with PEG-it virus precipitation solution (System Biosciences) based on manufacturer's protocol. Concentrated virus was resuspended in minimal volumes of cold PBS (100-150uL) to maximize virus concentration. Viral titer and concentration was measured using an HIV-1 p24 antigen ELISA (Fisher Scientific) following manufacturer's protocol. To optimize *in vivo* lentivirus infection, 1500ng/ml and 3000ng/ml virus have been used in addition to 8 µg/ml polybrene (Sigma-Aldrich) and 1x PBS (Kaufman et al., 2014).

In vivo viral infections have been performed in collaboration with the UCSD Transgenic Mouse Facility. To date, our lab has only done the CMV-GFP optimization experiments. Viral infection of blastocysts and embryo transfer were performed based on a previously published protocol (Kaufman et al., 2014). Females were sacrificed at e13.5 and embryos and placentas were dissected, weighed and observed under fluorescence dissecting microscope to determine if infection was successful and expressed specifically in the placenta. Samples were then fixed with 2% paraformaldehyde (PFA) in PBS overnight, exposed to a sucrose gradient (8% - 18% sucrose/PBS) and then embedded in OCT (Tissue Tek). 7µm sections were cut and stained with DAPI as a nuclear counterstain.

2.4 Immunostaining of cells and tissues

Tissue samples were fixed in either formalin or 4%PFA/PBS and then paraffin-embedded. All immunohistochemistry was performed using the Ventana Discovery

Ultra automated immunostainer (Vanata Medical Systems, Tucson, AZ). Standard antigen retrieval and reagents were used per manufacturer's protocol. Serial sections were stained with pan-cytokeratin (Z0622, Dako Cytomation), phospho-Histone3 (ab32107, Abcam), Sirt1 (07-131, Millipore), Ki67 or CD31. Glycogen trophoblasts were evaluated *in vivo* by Periodic Acid-Schiff (PAS) staining according to manufacturer's protocol (Sigma).

For immunofluorescence staining of cells, TS cells were seeded on glass coverslips placed inside standard tissue culture plates. At timepoints of interest, samples were fixed with 4%PFA for 10 minutes and then washed with PBS three times. Cells were then permeabilized with 0.5% Triton-X for 10 minutes, then rinsed with PBS three times for 5 minutes. Slides were incubated in blocking solution (5% Goat Serum, 1% BSA) for 1 hour at room temperature and then exposed to primary antibodies for 1 hour. After three 5 minute PBS washes, samples were then incubated with secondary antibodies for 1 hour in the dark and then washed again for 5 minutes in PBS (x3). Staining was done using the following dilutions of primary antibodies: 1:1000 Sirt1 (07-131, Millipore), and 1:500 b-Catenin (610154, BD). Alexa Fluor 488 or 594 conjugated (anti-mouse and anti-rabbit?) secondary antibodies were used (Invitrogen). Nuclear staining was done using 1:1000 dilution of DAPI (Invitrogen) while cell morphology was visualized using 1:500 A546-conjugated phalloidin (A22283, Invitrogen).

2.5 In situ hybridization

In situ hybridization (ISH) was also performed using the Ventana Discovery Ultra automated immunostainer (Vanata Medical Systems, Tucson, AZ). RNAscope ISH probes were purchased from ACD-Bio (Hayward, USA). These probes were

specific to SynA, Gcm1, Ctsq, Tpbpa, Prl2c2, Epcam, Rhox4, and Mash2. RNAscope probes are designed based on the z-DNA methodology where two adjacent 18-25-base pair (bp) probes hybridize to the mRNA sequence (Wang et al., 2012). The z-DNA probe has a 14-bp linker region and an adjacent 25-bp sequence; when two z-pairs hybridize next to each other, they contain a 50-bp backbone to which a subsequent probe and amplifier sequence hybridizes. This allows for up to 8000 labels for each target mRNA (Wang et al., 2012). Following amplification steps, the probes were visualized with 3,3'-diaminobenzidine (DAB) and slides counterstained with hematoxylin. Each of the probes used in the study were tested in tissues where their expression level and pattern could be readily validated. Both negative (bacterial *dapB* mRNA) and positive control probes (cyclophilin PPIB mRNA) were used on selected tissues to ensure probe specificity. ISH slides were analyzed by conventional light microscopy on an Olympus BX43 microscope (Olympus, Waltham, USA). Each dot corresponds to a single RNA message (mRNA); larger dots may be multiple mRNAs closely localized.

2.6 Flow Cytometry

Cell proliferation was evaluated using a BD Pharmingen BrdU Flow Kit (BD Biosciences). WT and Sirt1-null TS cells were treated with fresh growth media for 1 hour prior to BrdU pulse and then treated with 10 μ M BrdU for 30 minutes. Cells were then collected, stained and assessed by flow cytometry according to the manufacturer's protocol. Flow cytometry was also used to evaluate trophoblast apoptosis. To assess apoptosis, cells were first stressed using hypoxia-reoxygenation. WT and null TS cells were exposed to 2% oxygen for 24 hours and then re-exposed to normoxia for 1 hour before collection. Live cells were collected and stained for

Annexin V and 7AAD using the protocol and reagents of the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Epcam^{high} cells were measured using anti-mouse CD326 (Epcam)-PE antibody (clone G8.8, eBiosciences). Cells were differentiated for 5 days and then collected and stained with antibody diluted in flow cytometry buffer (1% BSA, 10% FBS, PBS) for 1 hour, washed twice in buffer, and the resuspended in 500ul buffer. Cells were immediately assessed by flow cytometry. All experiments were evaluated immediately using the FACS-Canto Flow cytometer.

2.7 Western blot analysis

Cells were lysed in standard protein lysis buffer (1% TritonX-100, 0.5% SDS in 1x Tris-buffered saline) plus fresh HALT™ Protease and Phosphatase Inhibitor Cocktail and EDTA (Thermo Scientific). Samples were then homogenized by sonication. The Pierce BCA protein assay (Thermo Scientific) was used to measure protein concentrations. 20-45ug total protein lysate was run on either 8% or 10% denaturing polyacrylamide gels. Next, proteins were transferred to a PVDF membrane and blocked for 1 hour in either 5%BSA/TBST or 5%Milk/TBST and then incubated overnight at 4 °C with primary antibody. Membranes were subsequently washed three times with TBST, exposed to secondary antibody for 1 hour at room temperature and then washed again with TBST. Blots were promptly imaged. The primary antibodies and dilutions used were: Cell Signaling antibodies (all at 1:1000) of Met (cat # 3127), phospho-cMet (Tyr1234/1235) (cat # 3077), phospho-cMet (Tyr1003) (cat # 3135), phospho-cMet (1349) (cat # 3133), phosphor-Smad2/Smad3 (ser423/425)(cat # 8828), Smad2/3 (cat # 3102), phosphor-Smad1/5/8 (cat # 9511), Smad1 (cat # 6944), phosphor-p38/MAPK (cat # 9211), p-38/MAPK (cat # 9212), phosphor-AKT (Thr308), phospho-AKT (Thr450), phosphor-AKT (ser473) (cat #

9271), AKT (cat # 9272) as well as 1:1000 Sirt1 (07-131, Millipore), 1:5000 anti β -actin (A5441, Sigma) and 1:2000 Epcam (LS-C356623, LifeSpan).

2.8 RNA isolation, quantitative reverse-transcriptase PCR and gene expression microarray

Total RNA was isolated from WT and Sirt1-null trophoblasts using the NucleoSpin RNA extraction kit (Macherey-Nagel) for general timecourses. All RNA extraction was performed according to the manufacture's protocol. RNA concentration was measured using a NanoDrop. iScript cDNA synthesis reactions (BioRad) was done using 500ng of isolated RNA. Resulting cDNA was diluted 1:5 with nuclease-free water. Quantitative real-time polymerase chain reactions (qRT-PCR) using SYBR Green (Life Technologies) was used to evaluate gene expression. Unless otherwise specified, relative mRNA expression levels were determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), normalized against mouse 18S rRNA, then calculated as fold change compared to undifferentiated WT day 0 (d0) samples. Primers used for qPCR are listed in Table 1.

For microarray experiments, RNA extraction was done using MirVana RNA isolation kit (Ambion). Two microarray data sets were produced. In the first experiment, RNA samples submitted were from differentiated WT and Sirt1-null timepoints day 0, 1, 5, 7 and for the second experiment, samples assessed were of 43E2 (WT cells #2) and 29B (Sirt1-null cells #2) day 0, 2, 5, 7. RNA concentrations were measured using Qubit (Life Technologies) and RNA integrity was evaluated using Agilent 2100 Bioanalyzer (Agilent). All samples were prepared for microarray using the TotalPrep-96 RNA Amplification Kit (Life Technology). Global gene expression profiling was performed on the MouseRef-8 v2.0 Expression Bead chips

Table 2.1 List of primers used for genotyping and qRT-PCR

Genotyping Primers		
Gene	Primer Name	Sequence
Sirt1-null	Sirt1-null F	TTC ACA TTG CAT GTG TGT GG
	Sirt1-null R	TAG CCT GCG TAG TGT TGG TG
	Sirt1-null R2	ATT TGG TAG GGA CCC AAA GG
Sirt1-mutant	Sirt1-mutant F	TGG AAG GAA AGC AAT TTT GGT
	Sirt1-mutant R	CTT TGT TGG CTG CAA ATT CA
Sirt1-Flox	Sirt1-floxed F	GGT TGA CTT AGG TCT TGT CTG
	Sirt1-floxed F2	
	Cre	AGG CGG AT TCT GAG TTC GA
	Sirt1-floxed R	CGT CCC TTG TAA TGT TTC CC
Cyp19-Cre	Cyp19-Cre F	GAC CTT GCT GAG ATTAGA TC
	Cyp19-Cre R	GAG AGA GAA GCA TGT TTA GCT GGC C
qRT-PCR Primers		
Gene	Primer Name	Sequence
m18s	Forward	CGC GGT TCT ATT TTG TTG GT
	Reverse	AAC CTC CGA CTT TCG TTC TTG
Blimp1	Forward	GCA AAG AGG TTA TTG GCG TGG
	Reverse	TTC ACG TAG CGC ATC CAG TT
Bmpr1a	Forward	CAC CAG AGG ATA CCT TGC CTT
	Reverse	CTG GGC TTT CGG TGA ATC CT

Table 2.1 List of primers used for genotyping and qRT-PCR, Continued

Gene	Primer Name	Sequence
Bmpr1b	Forward	TGG AGC AGG ACG AGA CAT ACA
	Reverse	CTC CAC GCC ACT TTC CCA TC
Bmpr2	Forward	TCT TGC CGT CTG GCT CAT TC
	Reverse	GCC GCA TTA TCT TCT TCC CCT
Bmp4	Forward	TGA TAC CTG AGA CCG GGA AG
	Reverse	CTG GAG CCG GTA AAG ATC CC
Cdx2	Forward	CCA GCT CTT TGC CTC TCT GT
	Reverse	TGC CTC TGG CTC CTG TAG TT
Ctsq	Forward	AAC TAA AGG CCC CAT TGC TAC
	Reverse	CAA TCC CCA TCG TCT ACC C
Cx31	Forward	GTG GGG TGA CGA GCA AAA AG
	Reverse	TTC TGG CGA TGC TTC CGT TC
Esrrb	Forward	GGG AGC TTG TGT TCC TCA TC
	Reverse	CTA CCA GGC GAG AGT GTT CC
Epcam	Forward	GGG CGA TCC AGA ACA ACG A
	Reverse	TGA TGG TCG TAG GGG CTT TC
Gcm1	Forward	GCC TGT GTT GAG CAG ACC TT
	Reverse	GGG AGT TGT GGT TGT TGG TG
Hes1	Forward	AAT ACA AAG GCG CAA TCC AA
	Reverse	AGG CCA CTG CTA CCC GTA AA

Table 2.1 List of primers used for genotyping and qRT-PCR, Continued

Gene	Primer Name	Sequence
Ly6e	Forward	GCA TGG AGC AAG TTC ATT CCC
	Reverse	GAC GCC ACA CCG AGA TTG AG
c-Met	Forward	GTG CCC GAA GTG TAA GTC CAA
	Reverse	ACT TGT CAG GAT AGG GGA CAG G
Mash2	Forward	AGA CTC ATG CCC ACC TCG TCT
	Reverse	CCA CCT TAC TCA GCT TCT TGT TG
Pcdh12	Forward	GAA CCT GAC CTG GAA GAA GGG
	Reverse	GTT GTA GCA TCG GGG GAA GA
Prl3b1	Forward	CCA ACG TGT GAT TGT GGT GT
	Reverse	TCT TCC GAT GTT GTC TGG TG
Prl2c2	Forward	TGA GGA ATG GTC GTT GCT TT
	Reverse	TCT CAT GGG GCT TTT GTC TC
Rhox4	Forward	GGG CCT AAG TCA GTG TGG GT
	Reverse	TGG CTT CAC TCA CAC CTA TCC A
Sca1	Forward	GGA TGG ACA CTT CTC ACA CTA AC
	Reverse	GCA GGT AAT TGA TGG GCA AG
Sirt1	Forward	GCA CCG ATC CTC GAA CAA
	Reverse	TCC CAC AGG AGA CAG AAA CC
SynA	Forward	CCC TTG TTC CTC TGC CTA CTC
	Reverse	TCA TGG GTG TCT TCTG TCC AA

Table 2.1 List of primers used for genotyping and qRT-PCR, Continued

Gene	Primer Name	Sequence
Tpbpa	Forward	CGG AGG GCT CCA ACA TAG AA
	Reverse	TCA AAT TCA GGG TCA TCA ACA A
Tssc3	Forward	CCG ACG AGA TCC TTT GCG AG
	Reverse	CAC CTT GAG GAT GGA GTG GA

(Illumina, San Diego, CA) at the VA/VMRF Microarray & NSG Core (UC San Diego, CA).

2.9 Analysis of gene expression profiling data

Data from the two microarray experiments were combined. The data was filtered for detection pValue < 0.01 and normalized using Robust Spline Normalization with the lumi package in R. COMBAT was used to eliminate variation due to batches of microarray runs. The Qlucore Omics Explorer (Lund, Sweden) was used to analyze the microarray gene expression data sets, including the preliminary analysis using the Principle Component Analysis (PCA) to explore global gene expression of both WT and Sirt1-null trophoblast. To identify genes statistically significantly changing during WT TS differentiation, I filtered variables by variance ($v=0.01$) and then applied a multi-group statistical analysis (ANOVA) ($q \leq 0.05$). This list of probes (probes statistically significantly changing during WT differentiation) was subjected to a CLICK analysis in the program EXPANDER (Sharan et al., 2003). CLICK is an unbiased analysis that clusters probes together based on expression patterns, without application of statistics or knowledge of samples. I then focused on clusters of genes with biological significance (increasing or decreasing with differentiation).

For comparison of WT and Sirt1-null TS cells, I first filtered samples by a variance ($v = 0.01$) and then applied a two-group statistical analysis ($q \leq 0.05$) by type (WT d0 vs. Sirt1-null d0). Probes that were either statistically significantly upregulated or downregulated in null d0 were selected and exported from Qlucore. I calculated the fold change and only probes with a fold change of 1.5 or higher were used for further analyses. Ingenuity Pathway Analysis (IPA) was performed to identify canonical

pathways enriched in each data set. Gene ontology analysis was done using METASCAPE (Metascape.org).

2.10 Statistical Analysis

All TS cell experiments (qPCR, migration assays) were done at least in biological triplicates. Unless otherwise stated, data presented are the mean $\pm$ S.D. of three biological replicates. Student's *t* test was performed between WT and Sirt1-null, and *p* values below 0.05 were taken to indicate a statistically significant difference between the indicated samples.

Chapter 2, in part, contains material that is currently being prepared for submission for publication of the material. Arul Nambi Rajan Kanaga, Soncin Francesca, Moretto-Zita Matteo, Iyer Pooja, Tache Veronique, Pizzo Donald, McBurney Michael, Parast Mana M. The dissertation author was the primary investigator and author of this material.

Chapter 3

Characterizing the Role of Sirt1 in
Trophoblast Stem Cell Maintenance
in Vitro

3.1 Sirt1 expression in mouse trophoblast stem (TS) cells

Mouse trophoblast stem (TS) cells can be isolated and cultured *in vitro* and can recapitulate all *in vivo* mouse trophoblast lineages. I first evaluated Sirt1 expression during standard differentiation in a wild-type TS cell line. Sirt1 RNA expression (Figure 3.1A) peaked after 1 day of differentiation and was downregulated d4-d7 of differentiation however no difference was detected in Sirt1 protein levels (Figure 3.1B).

Next, two wild-type (WT) and Sirt1-null trophoblast stem cell lines were derived to determine the role of Sirt1 in trophoblasts. Cell genotypes were confirmed by PCR (Figure 3.2B) and absence of Sirt1 was confirmed by western blot for Sirt1 (Figure 3.2C). Although Sirt1-null TS cells were derived and maintained, neither null cell line exhibited WT TS cell morphology. Null cell colonies were flatter, consisting of cells much larger than WT cells (Figure 3.2A). These cells also appeared to grow slower than WT counterparts.

3.2 Sirt1-null TS cells have defects in proliferation and apoptosis

Since Sirt1 plays a role in cell cycle, I evaluated proliferation of TS cells lacking Sirt1. Flow cytometry for Bromodeoxyuridine (BrdU), a marker for cells in S phase, and 7-AminoactinomycinD (7-AAD), as an indicator for total DNA, confirmed that fewer Sirt1-null TS cells were in S phase of the cell cycle (Figure 3.3A). Sirt1 is also widely recognized as a negative regulator of apoptosis (Vaziri et al., 2001; van der Horst et al., 2004) through targets such as p53. Phosphatidylserine (PS) is located on the cytoplasmic surface of cell membranes of healthy cells and moves to the outer membrane during early stages of apoptosis (Vermes et al., 1995). Since Annexin V has an affinity for PS, we used it as an indicator for onset of apoptosis.

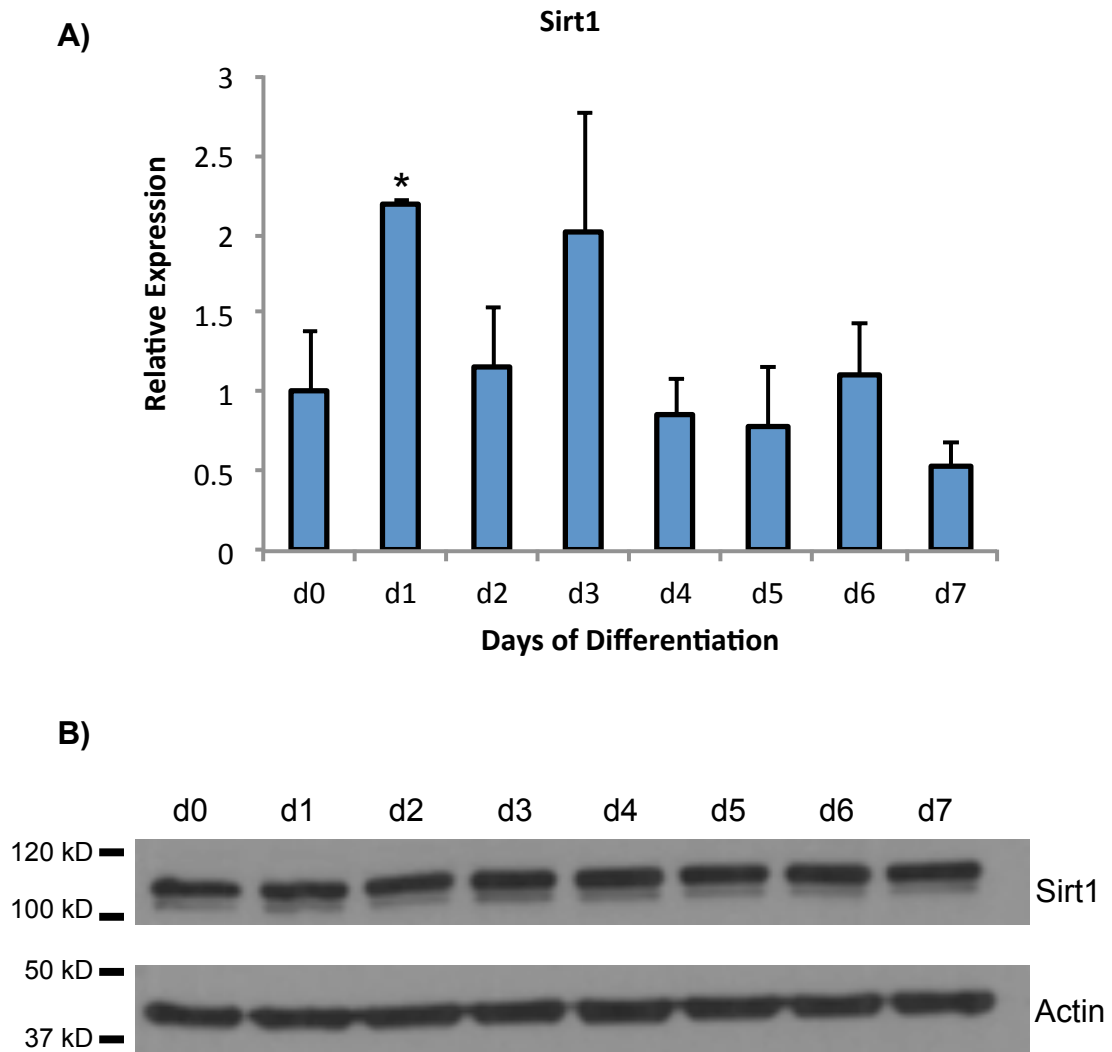


Figure 3.1 Sirt1 expression does not significantly change during mouse trophoblast stem cell differentiation

(A) qRT-PCR for Sirt1 RNA demonstrates a peak on d1 of in vitro trophoblast differentiation. (B) Western blot for Sirt1 did not detect any changes in Sirt1 protein expression. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

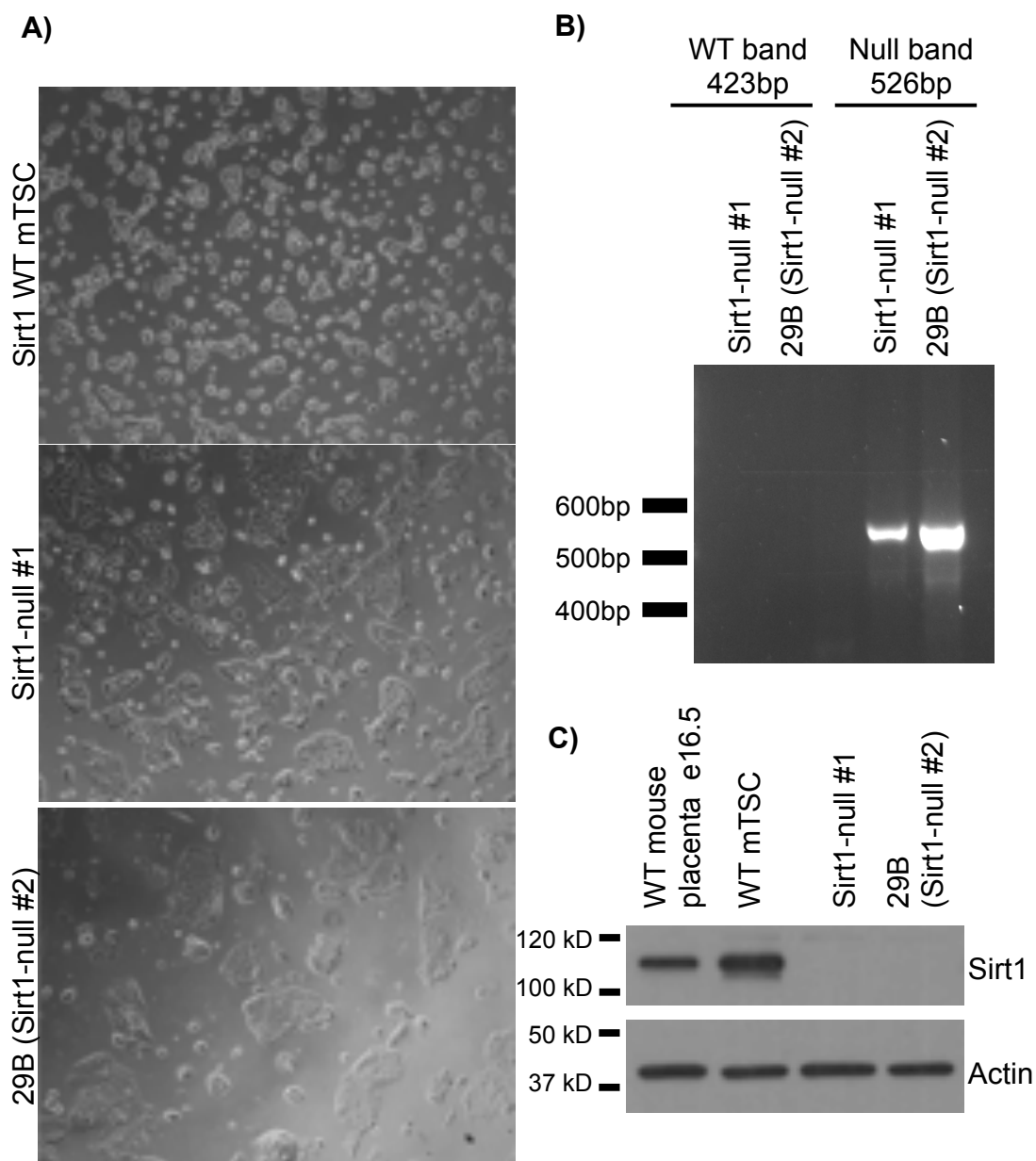


Figure 3.2 Isolation of Sirt1-null trophoblast stem (TS) cells

(A) Wild-type and Sirt1-null TS cells derived from e3.5 blastocysts. (B) Genotype of cells identified by PCR. (C) Western blot confirmed lack of Sirt1 protein.

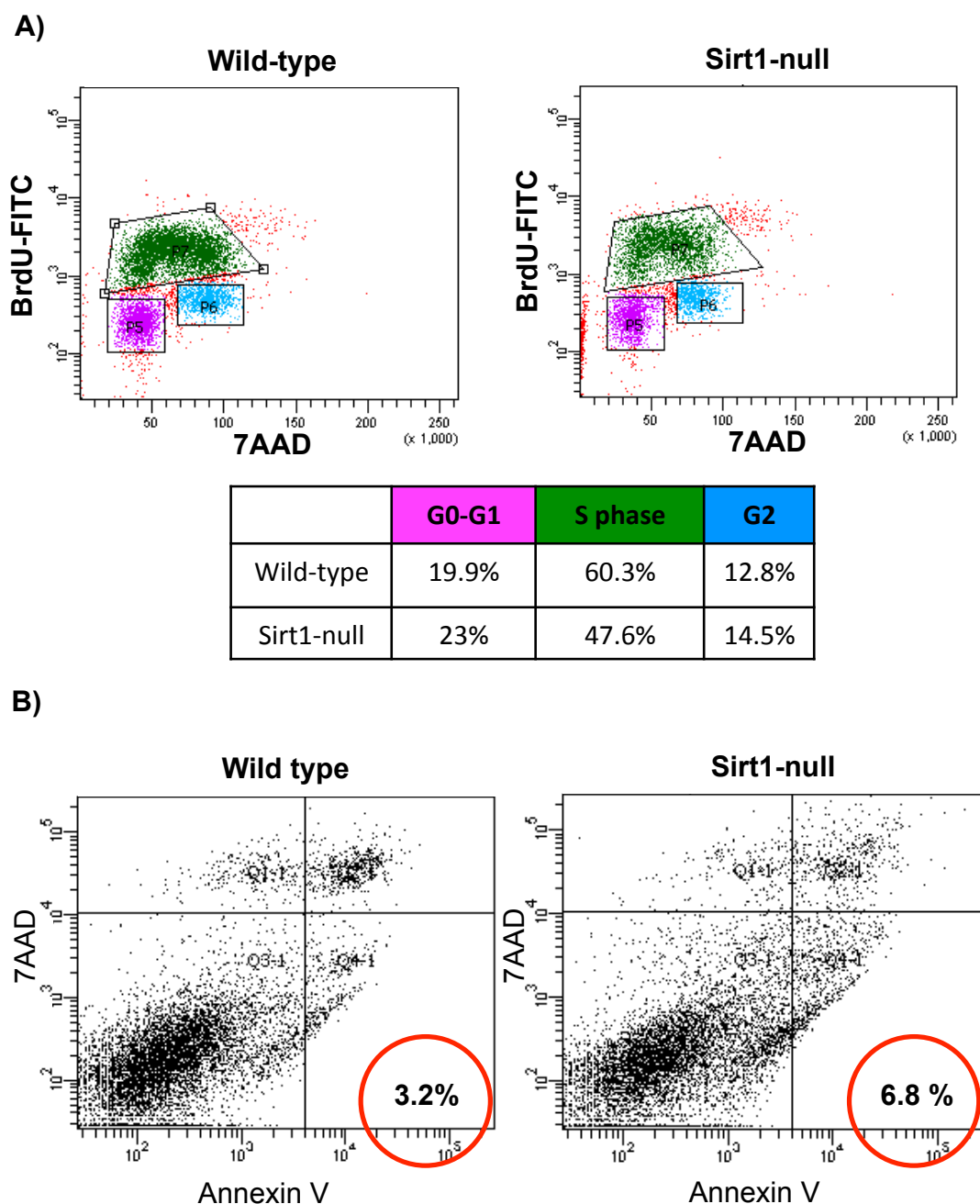


Figure 3.3 Reduced proliferation and increased apoptosis of Sirt1-null TS cells
 (A) Sirt1-null TS cells exhibit a decrease in proliferation based on BrdU-7AAD flow cytometry. (B) Compared to WT, null cells exhibit a larger proportion of Annexin V positive cells after being exposed to oxidative stress by 2% hypoxia-reoxygenation.

Undifferentiated trophoblasts were exposed to hypoxia for 24 hours and then re-introduced to normoxia oxygen conditions to induce oxidative stress. The reoxygenation resulted in an increase in the percentage of cells in early stage of apoptosis, and this population was two times higher in the absence of Sirt1 (Figure 3.3B).

3.3 TS cells lacking Sirt1 exhibit a differentiation defect

Due to the altered morphology and reduced proliferation of null TS cells, I hypothesized that Sirt1 plays a role in trophoblast differentiation and that TS cells lacking Sirt1 were more differentiated compared to WT TS cells. Indeed, undifferentiated null TS cells exhibited reduced expression of undifferentiated TS markers, *Esrrb* and *Cdx2*, based on quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (Figure 3.4A). However the expression of these markers decreased with differentiation in both WT and null TS cells. I also observed dysregulation of early and late (terminal) trophoblast differentiation markers.

Trophoblast stem cells in culture can differentiate into both labyrinthine and junctional zone cells (Oda et al., 2006; Soncin et al., 2015). However examination of early labyrinthine marker *Gcm1* and terminal marker *SynA* demonstrated reduced expression of labyrinthine lineage markers (Figure 3.4B). Given the reduced expression of these markers, I then examined expression of markers for the junctional zone lineages of spongiotrophoblasts, glycogen trophoblasts and trophoblast giant cells. *Tpbpa*, a marker of spongiotrophoblasts, *Cx31* and *Pcdh12*, markers of glycogen trophoblasts, and *PL-II* and *Ctsq*, markers of TGC, were all reduced in the null TS cells when compared to WT TS cells (Figure 3.5).

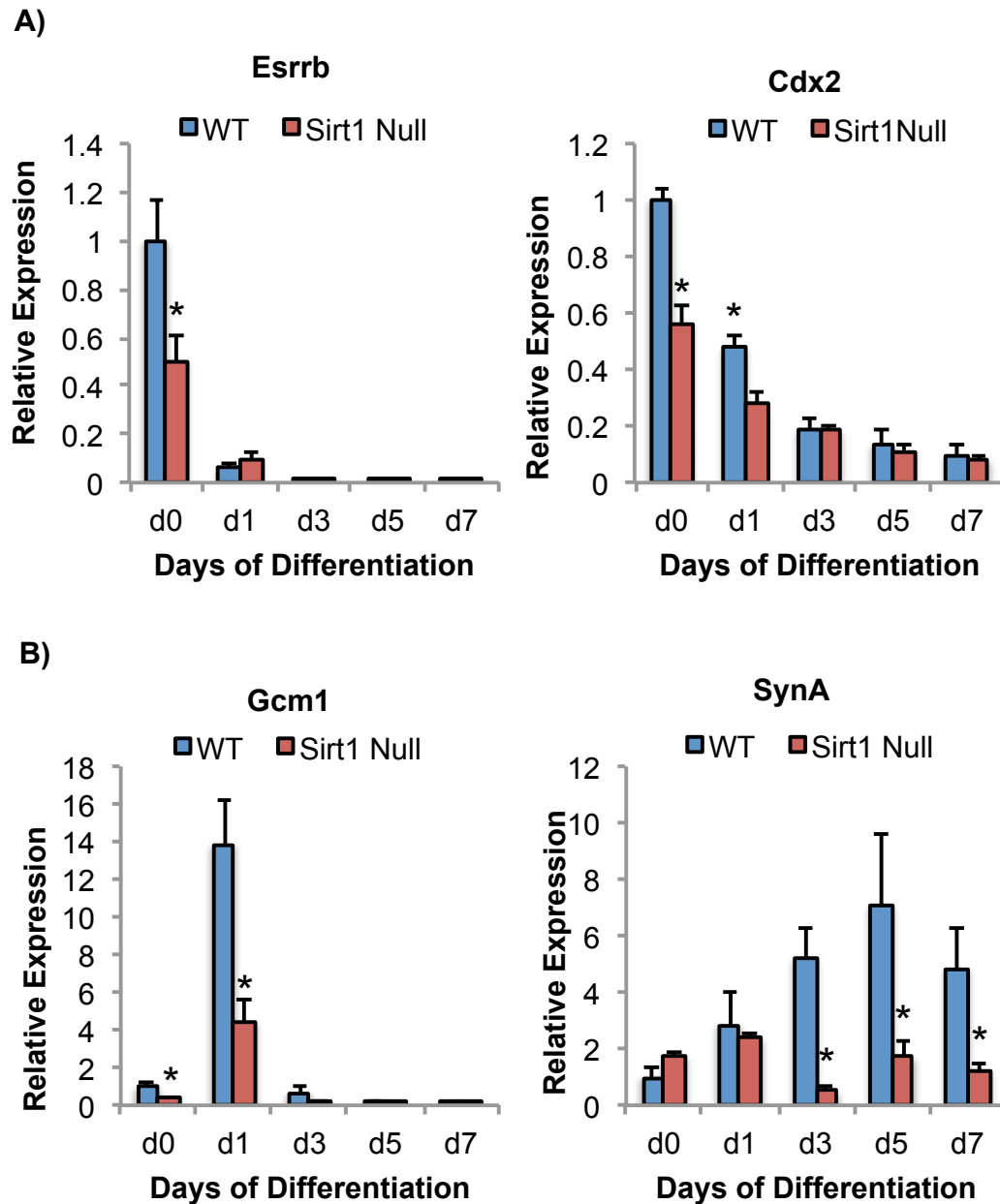


Figure 3.4 Reduced differentiation of Sirt1-null trophoblasts into labyrinthine cells

(A) In the absence of Sirt1, TS cells exhibit reduced TS markers Esrrb and Cdx2, by qRT-PCR. (B) However, these cells demonstrate reduced expression of early (Gcm1) and terminal (SynA) labyrinthine markers. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

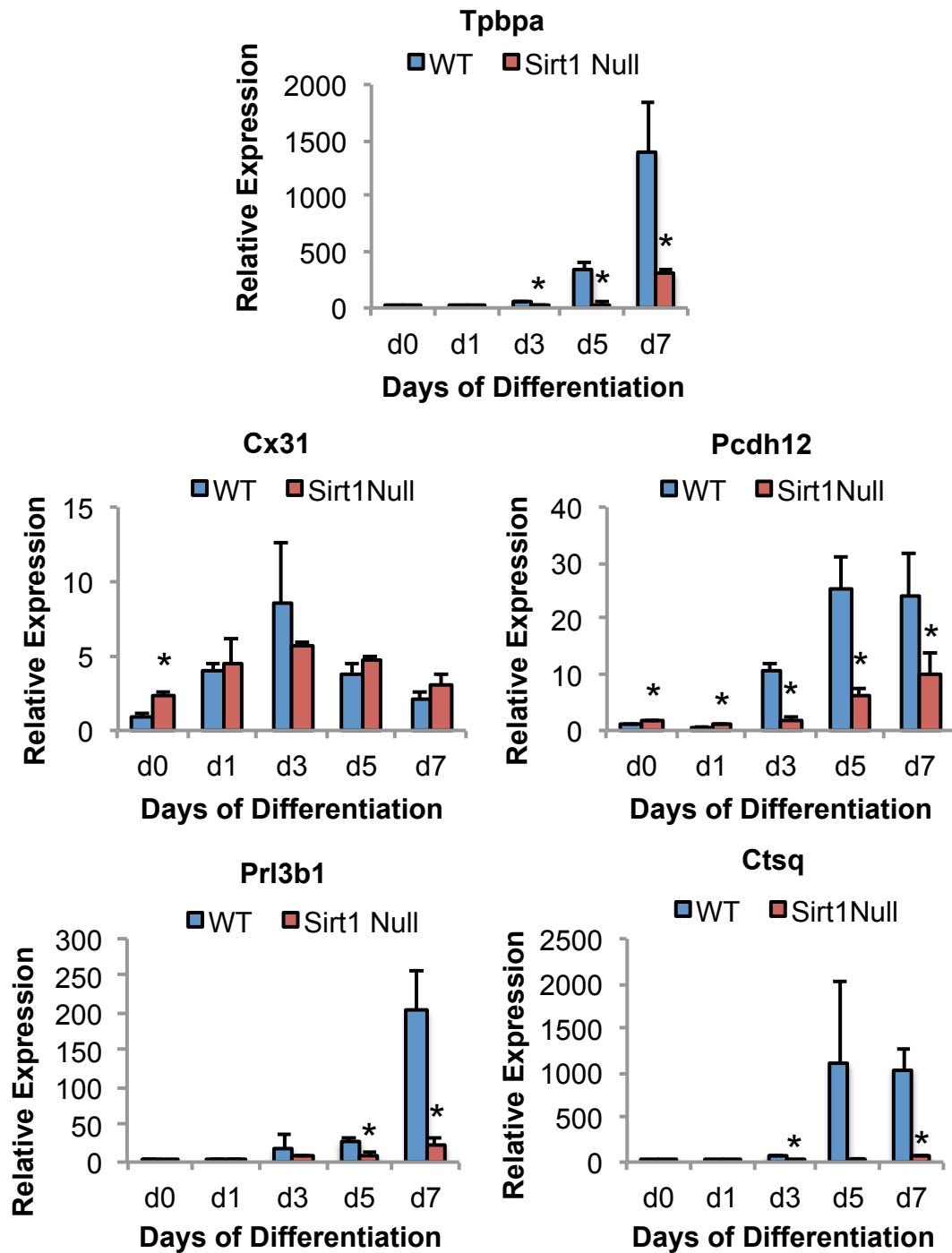


Figure 3.5 Null TS differentiation into invasive trophoblasts

Sirt1-null TS cells exhibit reduced expression of spongiotrophoblast (Tpbpa), glycogen cells (Cx31, Pcdh12) and trophoblast giant cell (Prl3b1, Ctsq) markers by qRT-PCR. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

To confirm that these observations were not due to cell line variability, I confirmed the differentiation defect in both an additional set of WT and Sirt1-null TS cell lines and in Sirt1-knockdown cell lines. New WT (cell line 43E2) and Sirt1-null (cell line 29B) were derived. Examination of select trophoblast lineage markers again demonstrated reduced TS cell, labyrinthine, spongiotrophoblast, glycogen and TGC markers (Figure 3.6). As a secondary confirmation, I knocked down Sirt1 expression in WT TS cells using five different Sirt1 shRNA constructs. Successful Sirt1 knockdown was determined by western blot (Figure 3.7A). Compared to the original WT cells, reduced expression of Sirt1 also resulted in reduced expression of differentiation markers *Esrrb*, *SynA* and *Prl3b1* by qRT-PCR (Figure 3.7B).

Next, I evaluated the differentiation defect of Sirt1-null TS cells at the functional level. Though there are no established functional assays for labyrinthine trophoblasts, invasive TGC differentiation can be confirmed by Matrigel invasion assays. Both WT and Sirt1-null TS cells were differentiated for seven days and then re-plated on a layer of Matrigel for the invasion assay (Figure 3.8A). Quantification of cells showed that in the absence of Sirt1, fewer differentiated trophoblasts invaded Matrigel (Figure 3.8B).

3.4 Sirt1-null TS cells are trapped in a progenitor state

Since all of the terminal trophoblast lineage markers are reduced in Sirt1-null TS cells, while TS markers are also reduced, I hypothesized that in the absence of Sirt1, TS cells are trapped in an intermediate trophoblast progenitor state. The different trophoblast lineages each stem from different populations of progenitor cells. Though a few of these progenitor populations have been identified, these cells are not well understood or characterized. *Sca1* is a marker for more undifferentiated

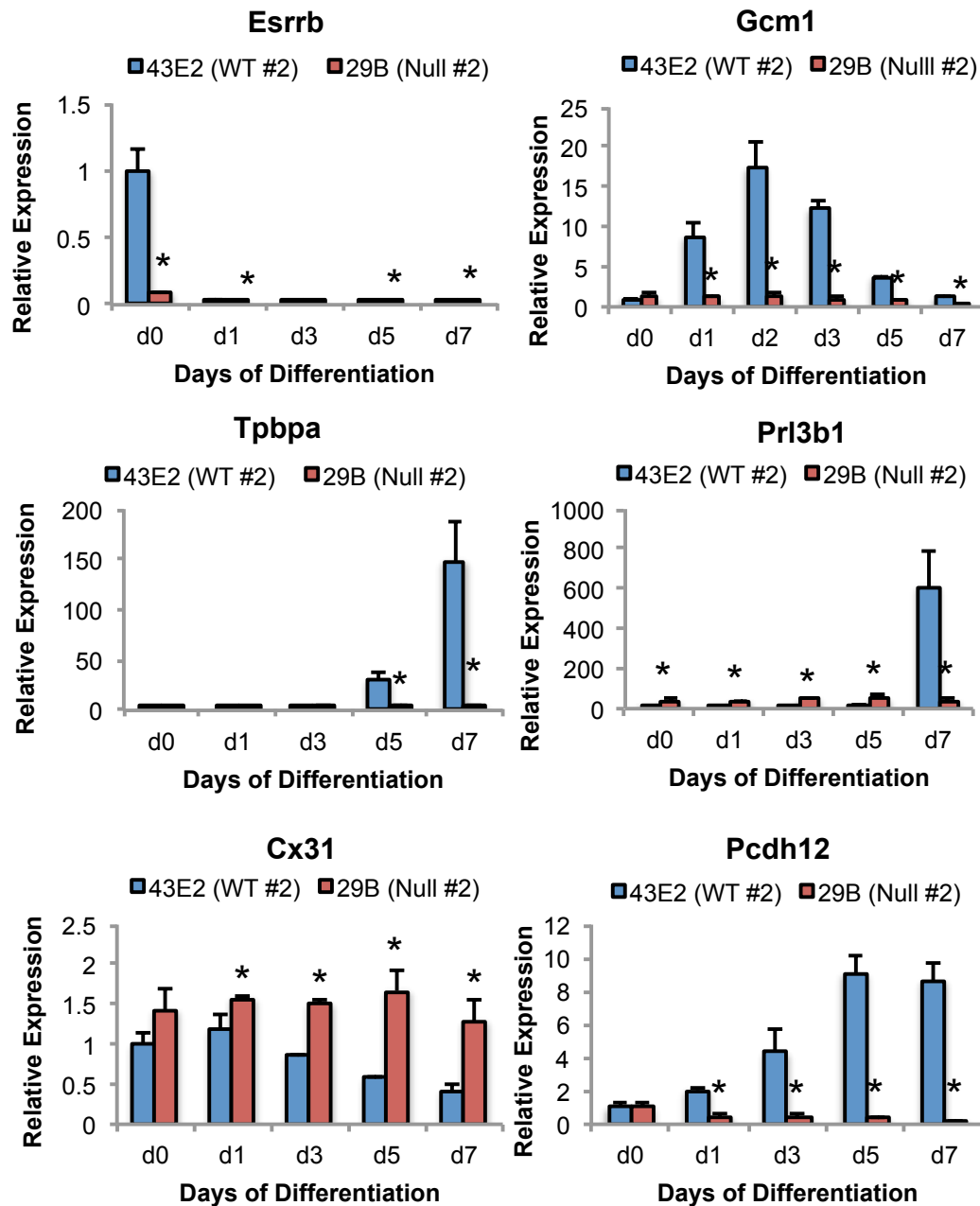


Figure 3.6 Sirt1-null TS differentiation defect confirmed in second WT and null TS cell lines

A second set of WT and Sirt1-null TS cells were isolated and differentiated. qRT-PCR for trophoblast lineage markers confirmed a differentiation defect of null TS cells into labyrinthine and invasive trophoblast lineages. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

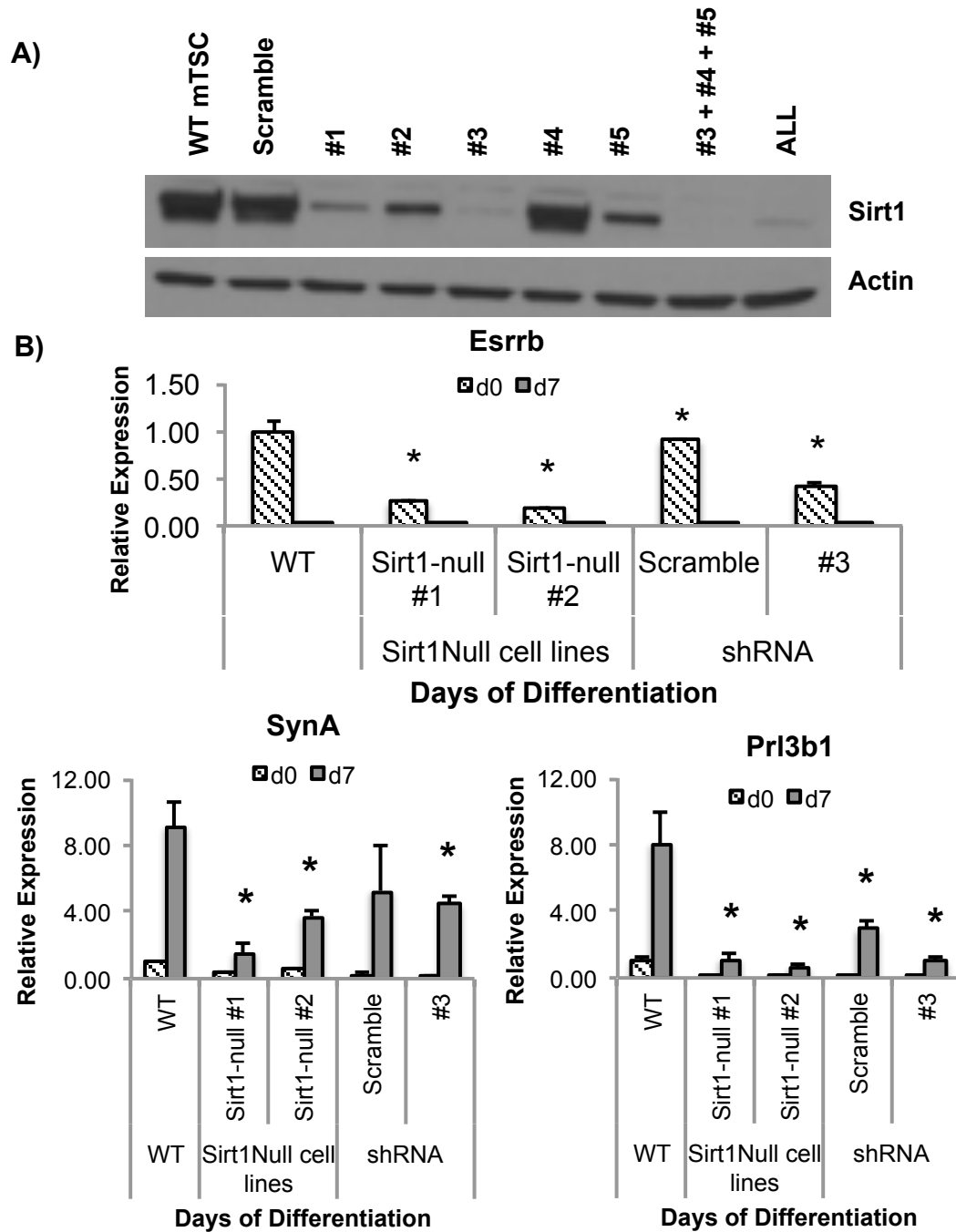


Figure 3.7 Sirt1 knockdown TS cells confirm null differentiation defect

(A) Sirt1 was knockdown in WT TS cells (line #1) by Sirt1 shRNA. Protein expression was detected by western blot. (B) TS knockdown lines also have reduced Esrrb on d0 but reduced SynA and PL-II by d7, detected by qRT-PCR. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$ compared to WT d0 (Esrrb) or WT d7 (SynA and PL-II).

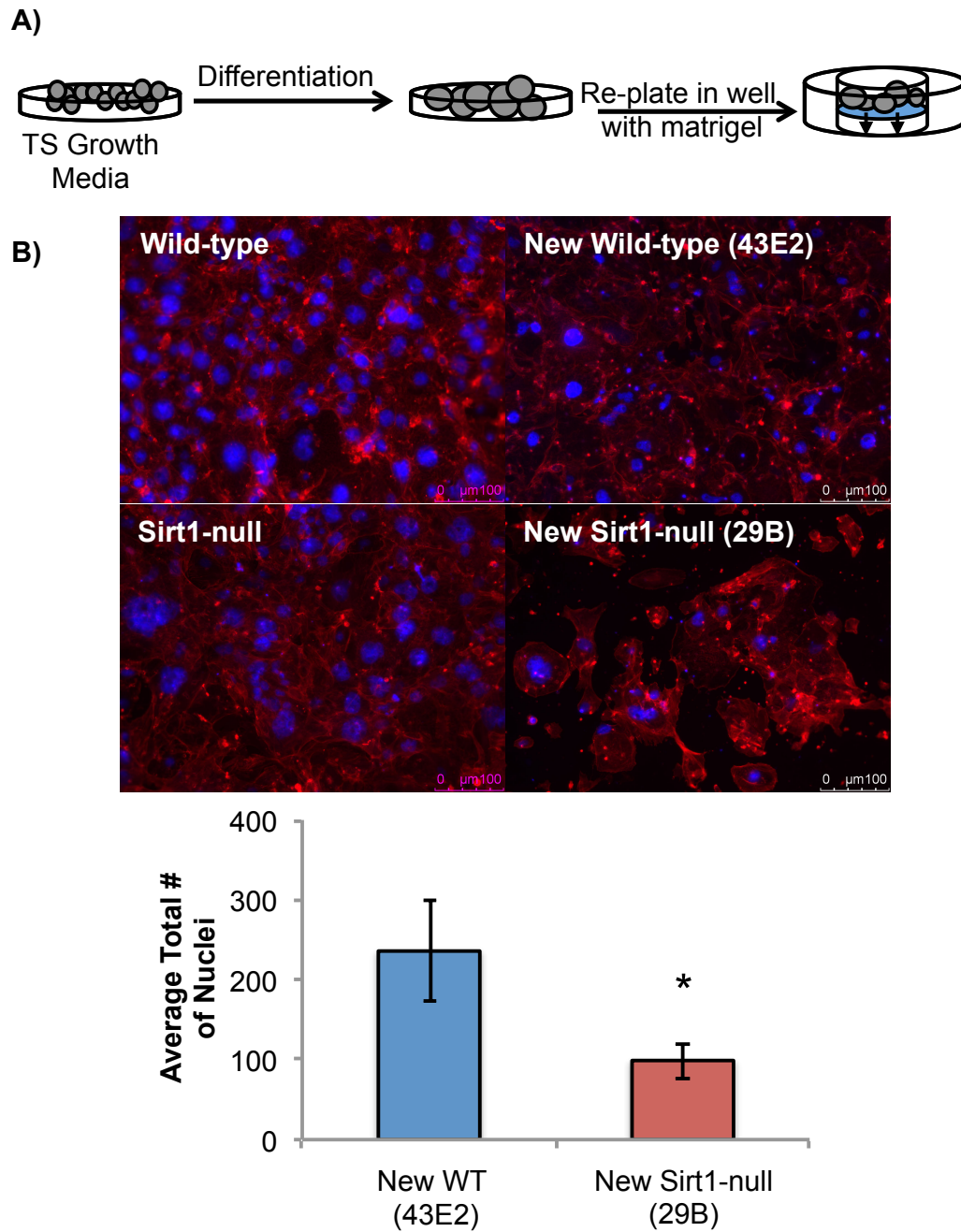


Figure 3.8 In the absence of Sirt1, cells have a defect in invasion

(A) WT and null TS cells were differentiated for 7 days and the plated on a layer of matrigel to study role of invasion. (B) Sirt1-null cells failed to invade at the same levels as WT cells. Cells were stained Phalloidin and DAPI. Nuclei were quantified. * $p < 0.05$.

trophoblasts (David Natale, unpublished observations). Though expression of Sca1 is lower in null d0 cells, it decreased in both WT and null TS cells (Figure 3.9A). More recently, Blimp1 was discovered as a marker of progenitors of invasive trophoblasts (Mould et al., 2012). Blimp1, similar to terminal invasive trophoblast markers, was downregulated in null TS cells (Figure 3.9B). Tssc3 was identified as a marker of spongiotrophoblast progenitor cells that give rise to Mash2 positive cells (Salas et al., 2004; Takao et al., 2012). Both Mash2 and Tssc3, though slightly higher in null trophoblast at a few timepoints, were not consistently different between WT and null TS cells (Figure 3.9C).

Several labyrinthine progenitors have been characterized as well. Ly6e is a general marker of labyrinthine progenitors that may give rise to precursors of SynT-I (Hughes et al., 2013) while Rhox4 is considered a marker of progenitors to SynT-II (Watson et al., 2011). Interestingly, Epcam has been identified as a marker of multipotent labyrinthine progenitor cells, with the ability to differentiate into SynT-I, SynT-II and S-TGC (Ueno et al., 2013). Ly6e, like terminal labyrinthine makers, is expressed at lower levels in TS cells lacking Sirt1, while both Rhox4 and Epcam exhibit an increase in Sirt1-null trophoblasts compared to WT (Figure 3.10). The high expression of Epcam RNA in null TS cells was confirmed in the second (29B) null TS cell line while other progenitor markers showed similar levels to WT cells (Tssc3, Mash2, Ly6e) or produced inconsistent results between the two null cell lines (Blimp1) (Figure 3.11). Flow cytometry of WT and null trophoblasts differentiated for five days demonstrated that though both WT and null trophoblasts exhibit Epcam-positive cells, there is a larger proportion of Epcam^{high} cells in the sample of differentiated Sirt1-null trophoblasts (Figure 3.12).

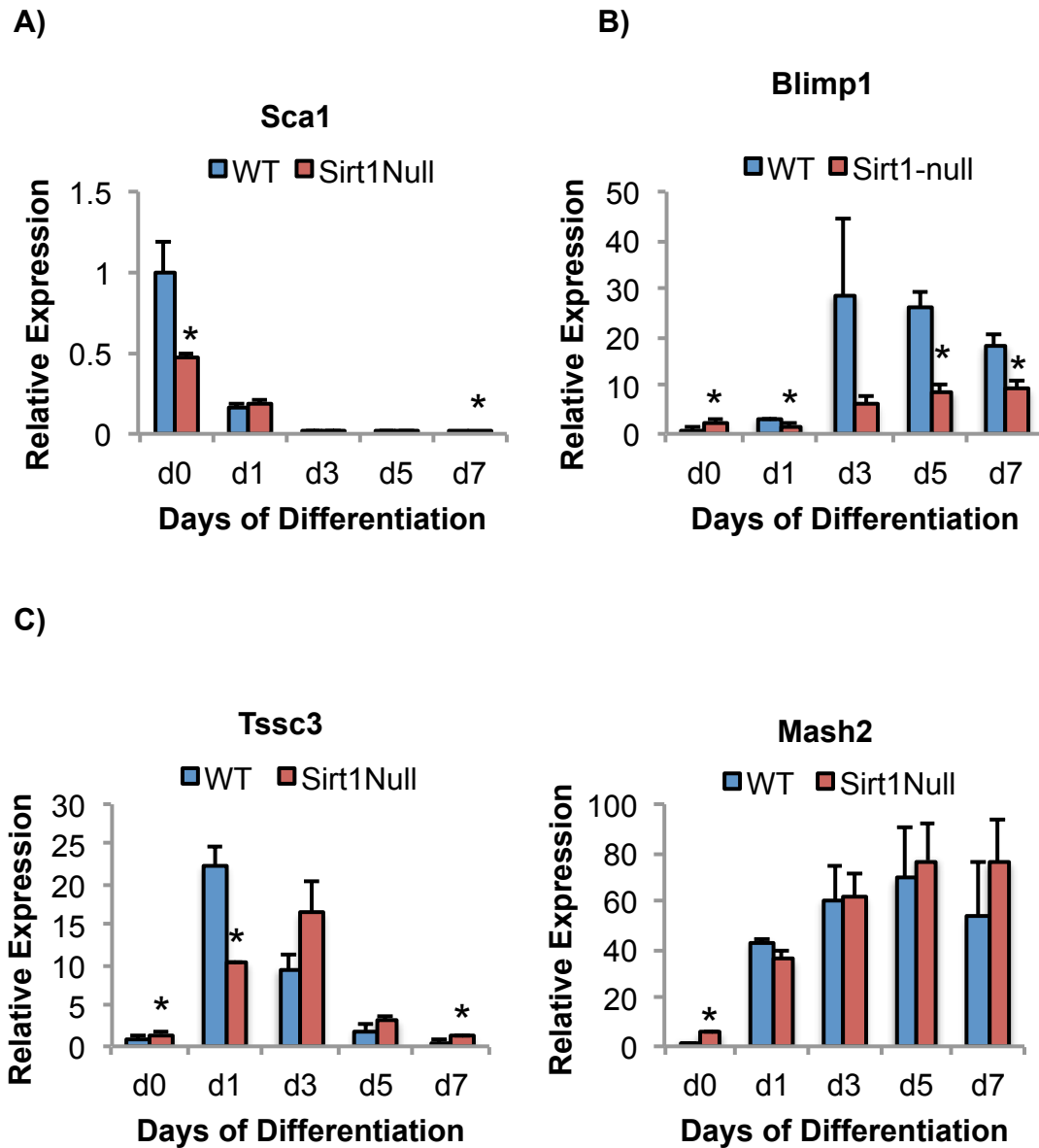


Figure 3.9 Expression of trophoblast progenitor markers

(A) Sca1 expression is low on in null cells d0 but is not different d1-d7. (B) Invasive trophoblast progenitor marker Blimp1 is reduced in null cells. (C) Tssc3 and Mash2, spongiotrophoblast progenitor markers, are not differentially expressed in WT and null trophoblasts. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

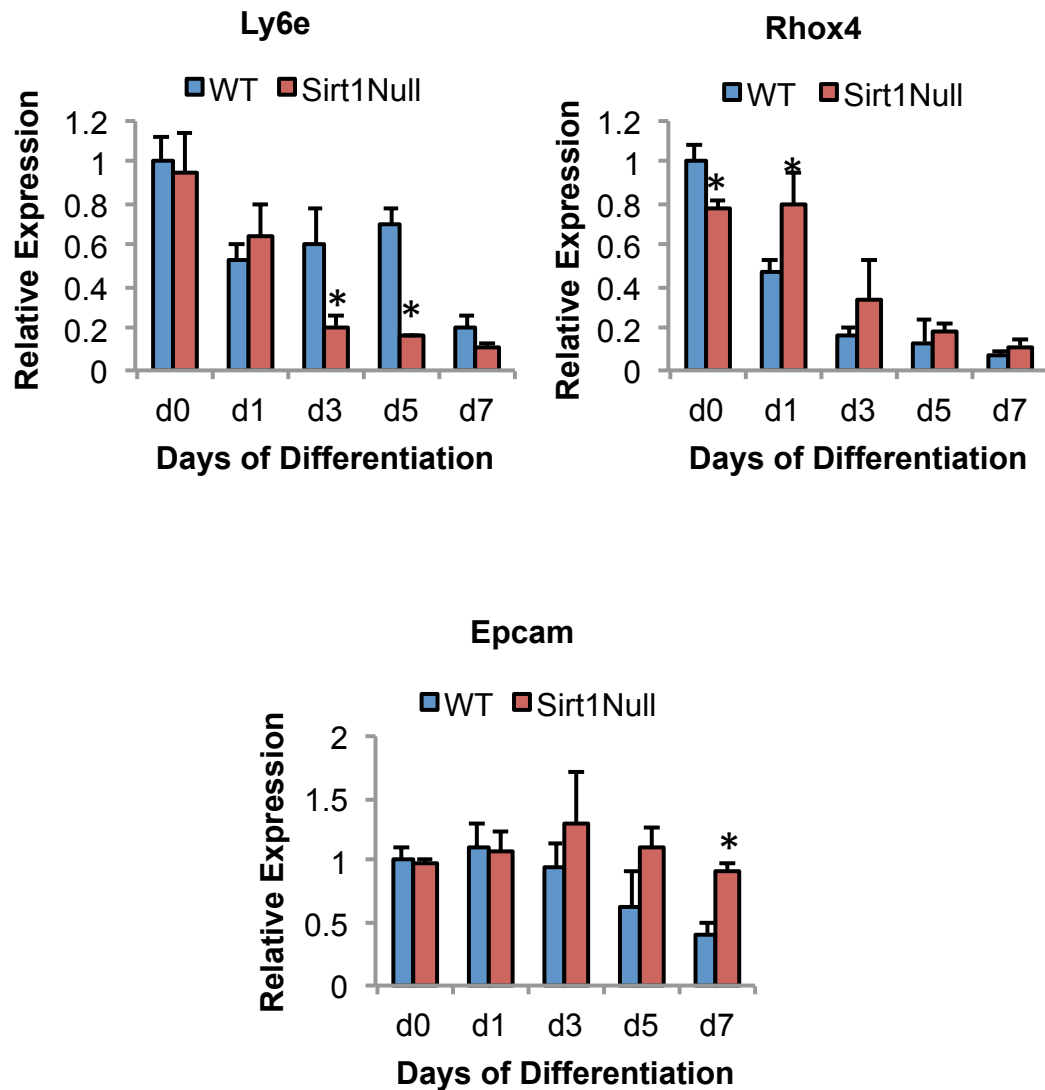


Figure 3.10 Expression of labyrinthine trophoblast progenitor

There are three known labyrinthine progenitor makers known. qRT-PCR of these markers showed differential expression in null trophoblasts. Ly6e is reduced in null cells and Rhox4 is slightly elevated in null trophoblast. Epcam is higher in the null cells at later differentiation timepoints. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

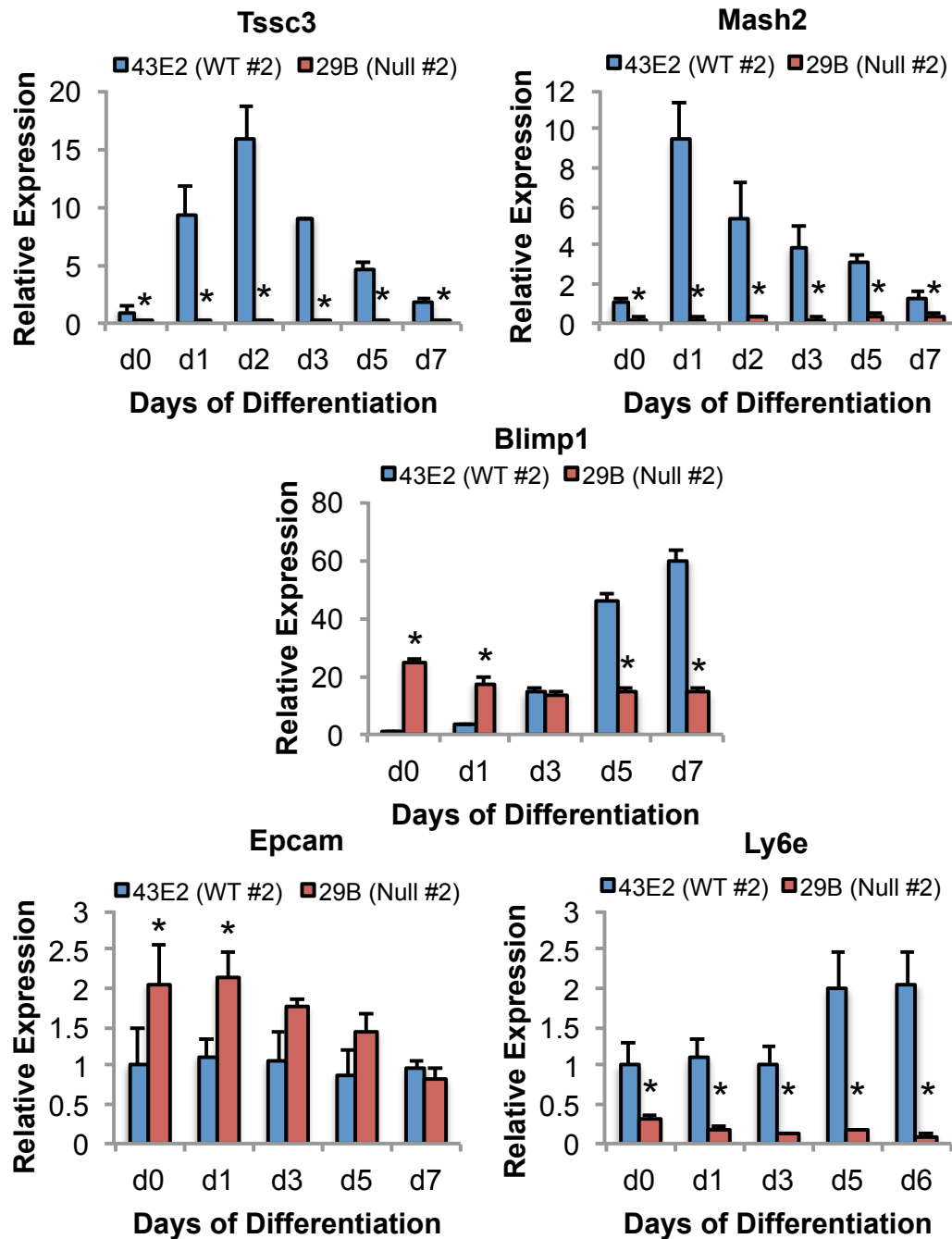


Figure 3.11 Trophoblast progenitor markers in second WT and Sirt1-null TS cell lines

qRT-PCR for markers of spongiotrophoblast and invasive trophoblast progenitors were downregulated in a second null cell in (29B) as was the labyrinthine progenitor marker, Ly6e. Only Epcam was upregulated. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

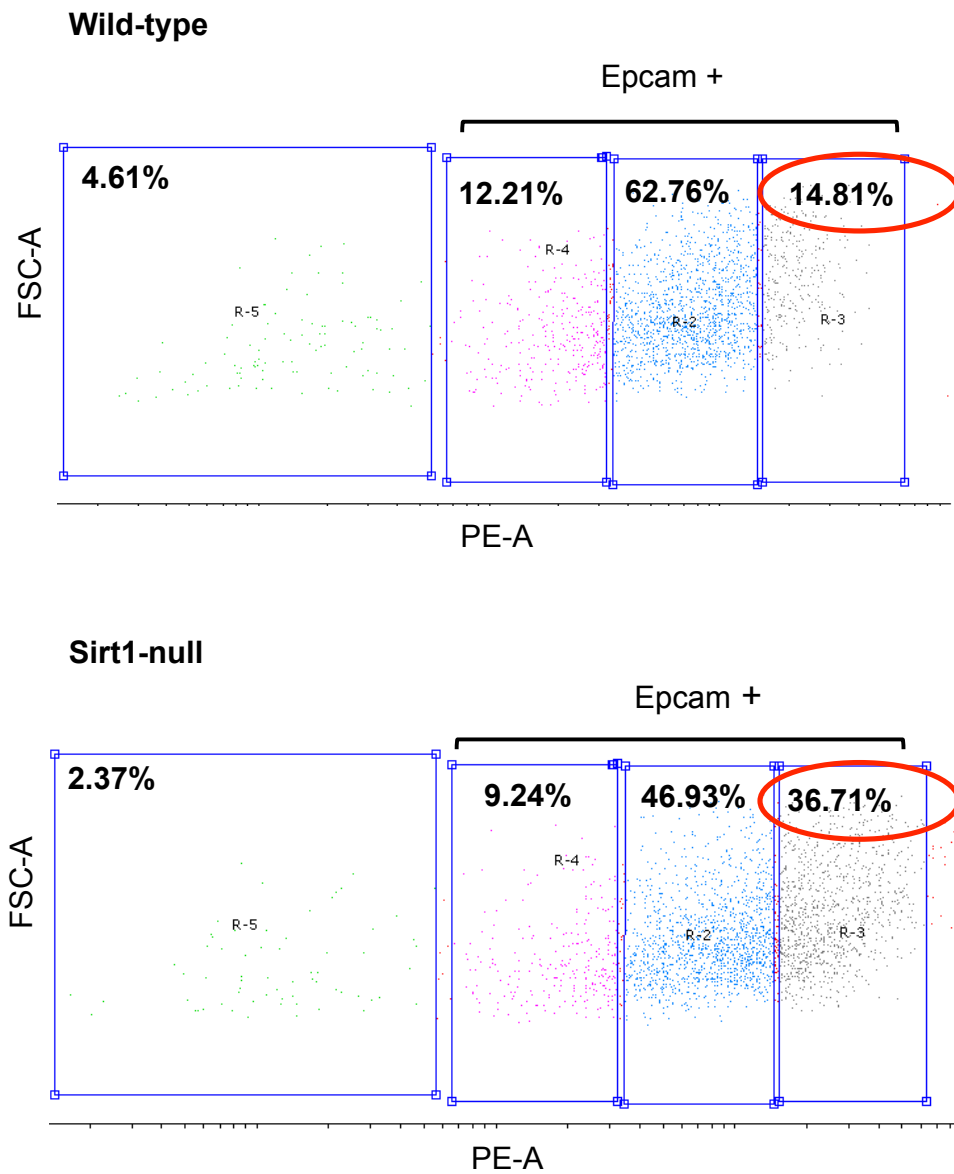


Figure 3.12 Increase in Epcamhigh cells in Sirt1-null trophoblasts

Flow cytometry of WT and Sirt1-Null trophoblasts differentiated for 5 days. Null trophoblasts exhibit a larger proportion of Epcamhigh cells compared to wild-type trophoblasts.

Chapter 3, in part, contains material that is currently being prepared for submission for publication of the material. Arul Nambi Rajan Kanaga, Soncin Francesca, Moretto-Zita Matteo, Iyer Pooja, Tache Veronique, Pizzo Donald, McBurney Michael, Parast Mana M. The dissertation author was the primary investigator and author of this material.

Chapter 4

Role of Sirt1 in Placental
Development and Intrauterine
Growth Restriction *in Vivo*

4.1 Sirt1 expression in the placenta

Due to ethical and technical issues, studying human placental development *in vivo* is difficult. For this reason, the mouse placental model has become critical in studying *in vivo* placental development. In mouse, the placenta can be divided into several main zones (Figure 4.1). Closest to the fetal surface is the labyrinthine layer. This layer is made up of three different trophoblast lineages (syncytiotrophoblasts layer I and layer II as well as sinusoidal TGC) in addition to fetal vasculature. These cells together facilitate gas and nutrient exchange between maternal and fetal blood. Above the labyrinth, approaching the uterine wall is the junctional zone. This layer is composed of Tpbpa-positive spongiotrophoblasts and glycogen cells. The junctional zone is close to the invasive giant cells that invade the maternal decidua. In mouse, Sirt1 is widely expressed throughout the placenta in nuclei of labyrinthine, spongiotrophoblast and trophoblast giant cells (Figure 4.2). However, expression in the TGC and spongiotrophoblast varies and Sirt1 is not expressed in all of these cells. Conversely, Sirt1 appears to be expressed in almost all cells of the labyrinth.

4.2 Characterization of Sirt1-null embryos and placentas

Sirt1-null mice on the 129/Sv background are growth restricted with observable developmental defects and die soon after birth (McBurney et al., 2003). We did not observe many homozygous Sirt1-null pups born (only 1 Sirt1-null pup compared to 661 pup at 10 year old pups). In our hands, null pups were embryonic lethal following e13.5 (Figure 4.3A). Similar to previous reports, these embryos were consistently growth restricted as indicated by 39% decrease in embryo weight; however, we also noticed a 16% decrease in placental weight as well (Figure 4.3B).

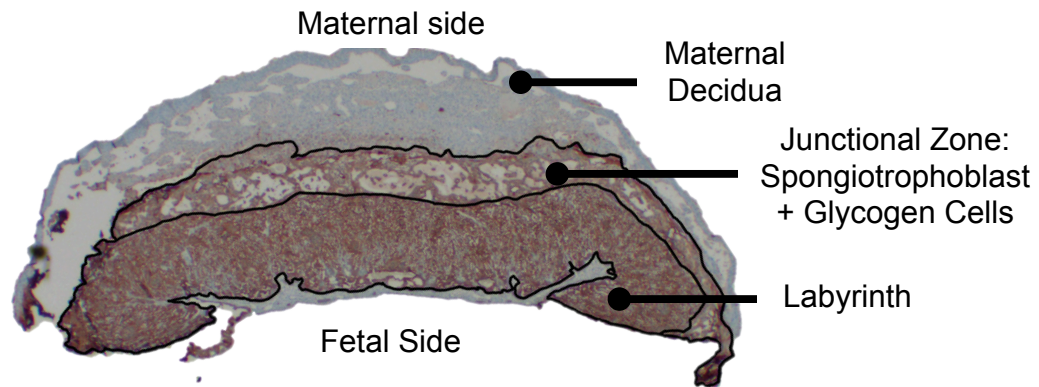


Figure 4.1 Schematic of e13.5 mouse placenta

Pan-cytokeratin staining to detect all trophoblasts in the an e13.5 mouse placenta. The three main zones of the placenta are the maternal decidua, the junctional zone and the labyrinth.

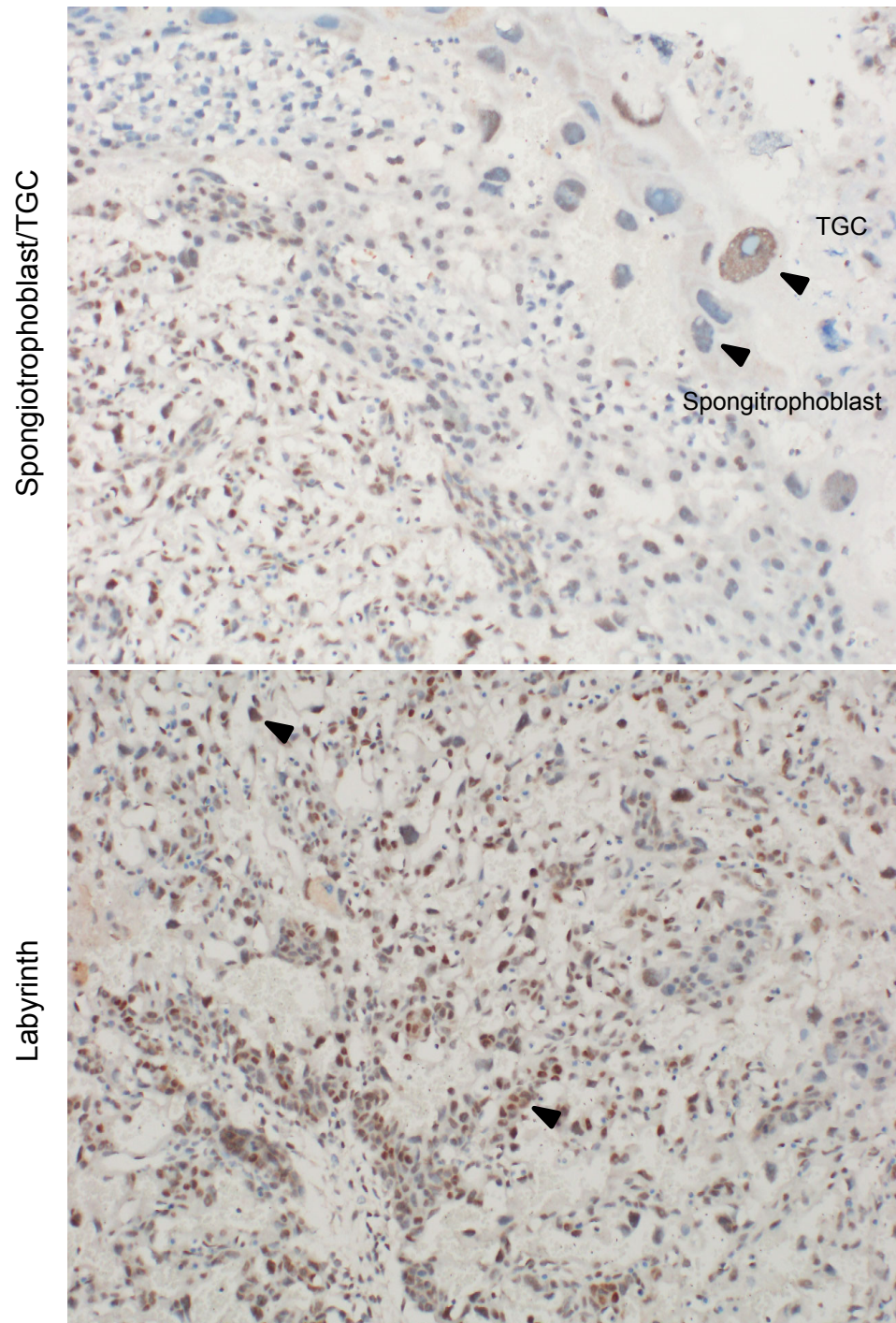


Figure 4.2 Sirt1 is strongly expressed in mouse placenta labyrinth

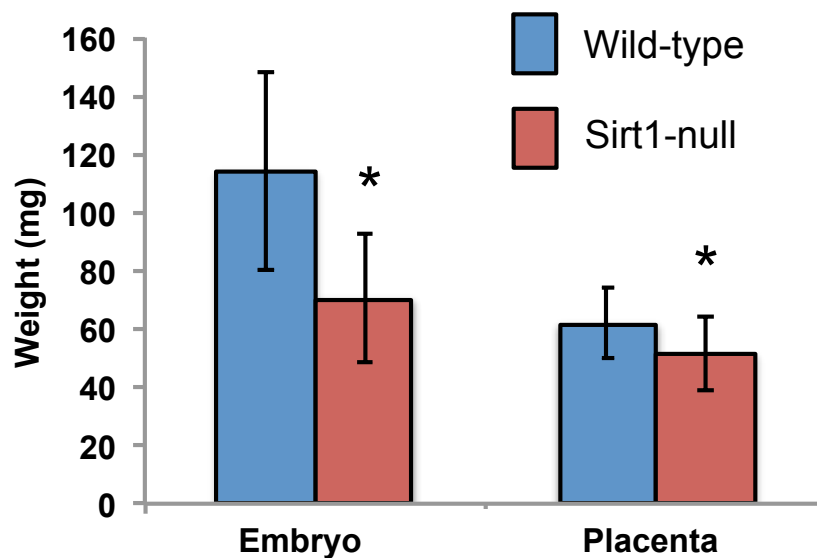
Immunohistochemistry for Sirt1 in a e13.5 mouse placenta. Nuclear Sirt1 was observed in all trophoblast layers but appeared to be expressed more highly in the labyrinth. Magnification is 200x and 400x.

A)

Pups Observed during Development

	WT	Het	Null
E12.5	2	5	1
E13.5	20	38	11
E14.5	16	13	3
E15.5	4	8	1
E16.5	3	1	1
10 day old pup	255	406	1

B)

**Figure 4.3 Sirt1-null embryos and placentas are growth restricted**

(A) Sirt1-null embryos are embryonic lethal around e13.5. (B) Both mouse placentas and embryos were growth restricted, as indicated by a 39% decrease in embryo and 16 % decrease in placental weight.

To identify potential etiologies for this reduced placental weight, I evaluated the Sirt1-null placenta morphology in more detail.

H&E staining of WT and Sirt1-null e13.5 placentas demonstrated morphological defects in null placentas. Compared to WT, placentas lacking Sirt1 exhibited a hypercellular labyrinthine layer (Figure 4.4). I hypothesized that this could be due to an increase in proliferative cells in the labyrinth. However both Ki67 (Figure 4.5A) and phosphorylated histone 3 (Figure 4.5B) immunohistochemistry did not demonstrate an increase in the number of proliferative cells in any placental compartment. Since null placentas weighed less, I also stained WT and null placentas with a general trophoblast marker, pan-cytokeratin, (Figure 4.6A) and measured the placental area (Figure 4.6B); however, no differences were detected.

4.3 Evaluation of labyrinthine trophoblasts in Sirt1-null placentas

Given the hypercellular morphology of the labyrinthine layer of the null placentas, I further evaluated the cellular components of the labyrinthine layer of null placentas. First, I stained WT and null placentas for CD31 to measure the area of the labyrinth (Figure 4.7A). However, no difference was detected in the labyrinthine area when CD31-positive staining area was normalized to total placental area (Figure 4.7B).

Next, I evaluated the labyrinthine trophoblast, of which there are 3 different sublineages. Different lineage-specific markers can identify each of these layers. Syncytiotrophoblast layer 1, layer 2 and sinusoidal TGC express SynA, Gcm1 and Ctsq (Simmons et al., 2008; Watson et al., 2011) respectively. I therefore performed *in situ* hybridization of e13.5 placentas for each of these markers to determine if there is any alteration of any labyrinthine layer (Figure 4.8). I noted an increase in SynA

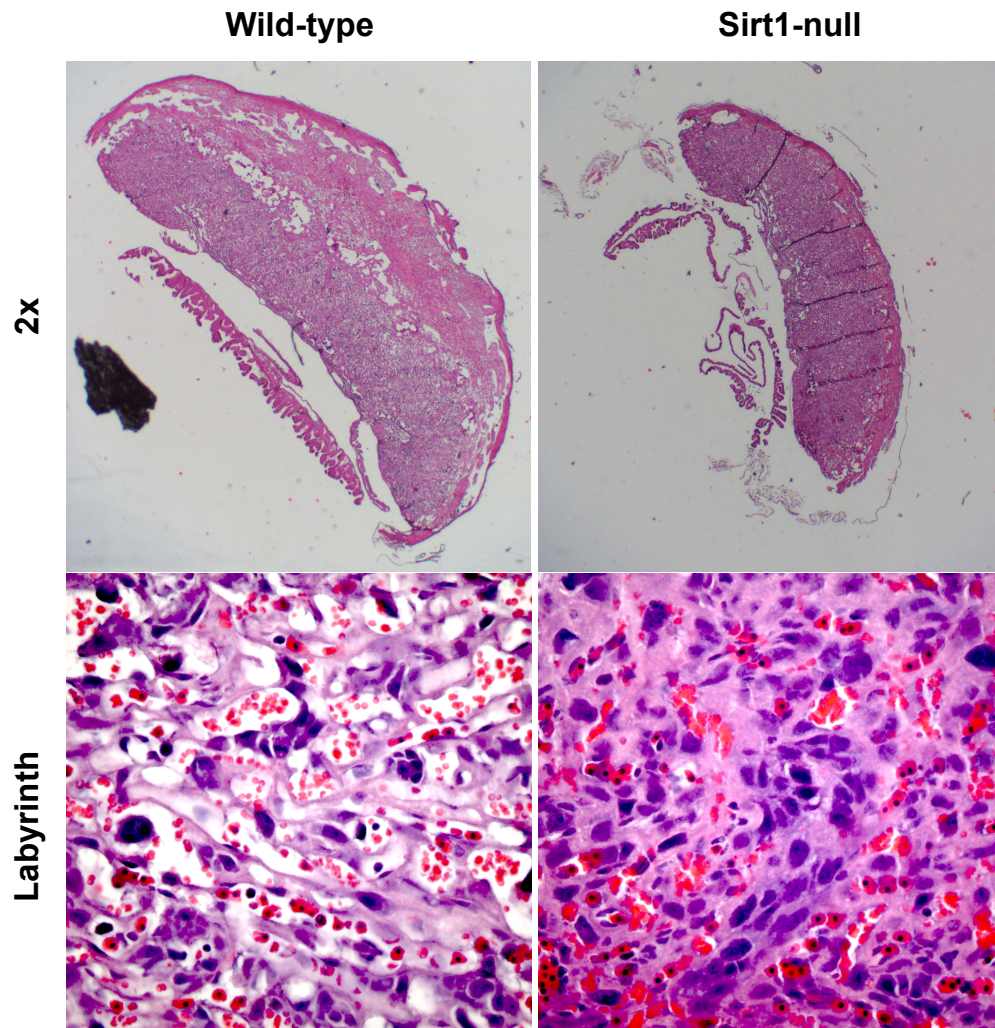


Figure 4.4 Sirt1-null placental labyrinth is hypercellular

H&E of e13.5 placentas showed that labyrinth morphology was altered in null placentas. Specifically, null placentas were noticeably hypercellular. Magnification is 20x (top).

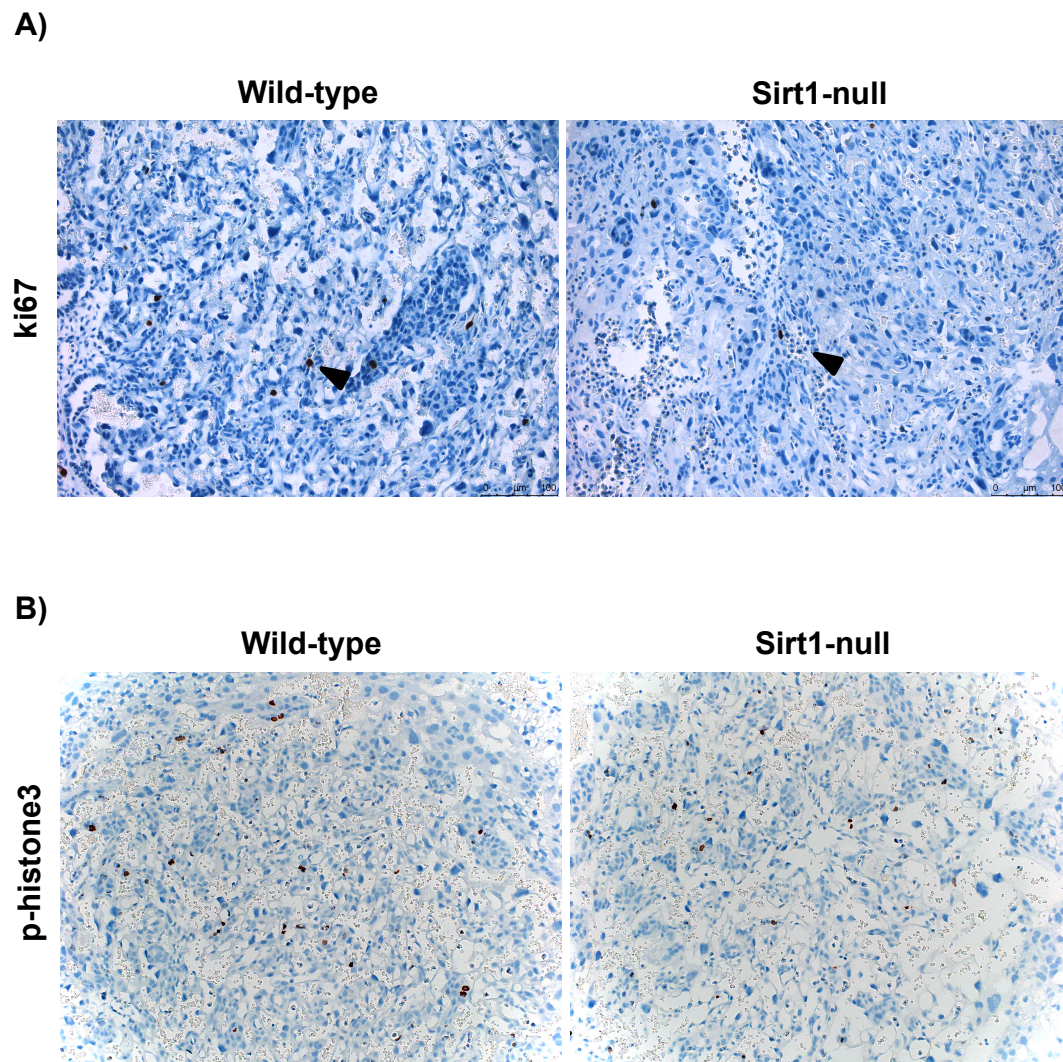


Figure 4.5 No difference in proliferative cells in WT and Null placentas
Labyrinth of e13.5 WT and Sirt1-null placentas stained with either proliferation markers ki67 (A) or p-histone3 (B). Magnification 200x.

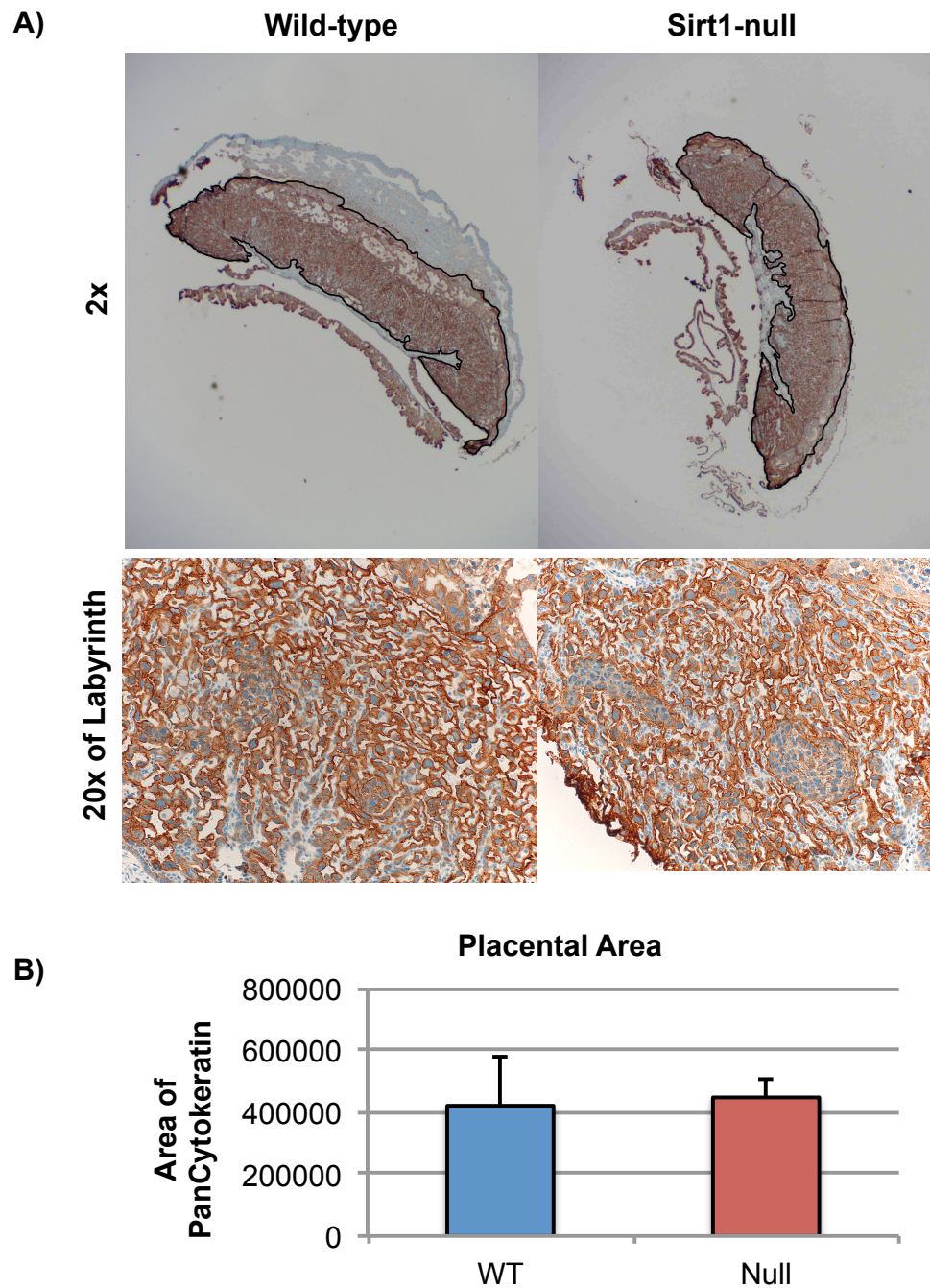


Figure 4.6 Measurement of total placental area

(A) e13.5 mouse placentas stained for Pan-cytokeratin identified all mouse placentas. Magnification is 20x (top) and 200x (bottom). (B) Area of stained region was measured for both WT and null placentas but no difference was detected.

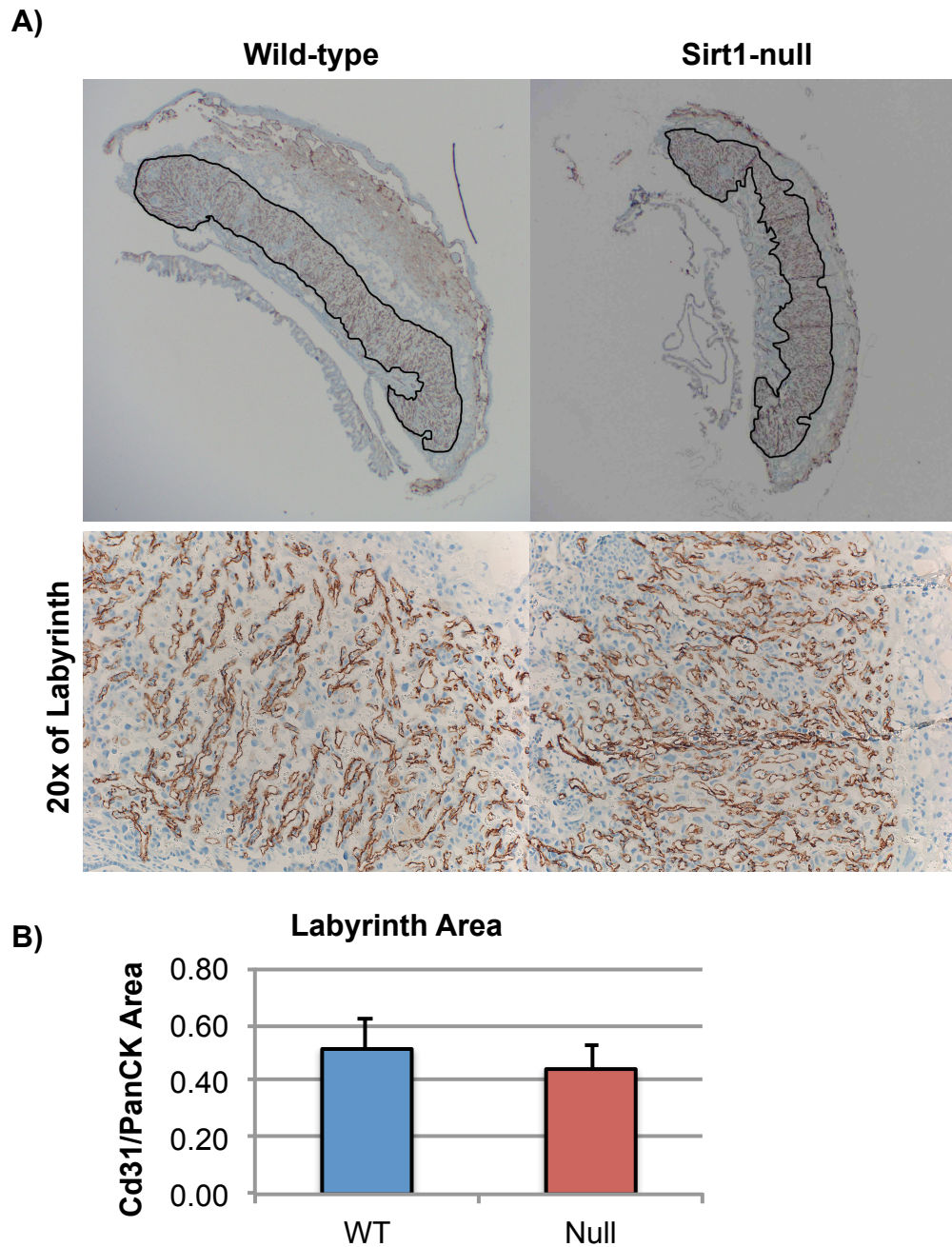


Figure 4.7 Evaluation of labyrinth area

(A) CD31 IHC was done to stain placental vasculature of e13.5 placentas. Magnification 20x (top) and 200x (bottom). (B) The area of CD31 positive staining was normalized to total placental area but no difference was detected between WT and null placentas.

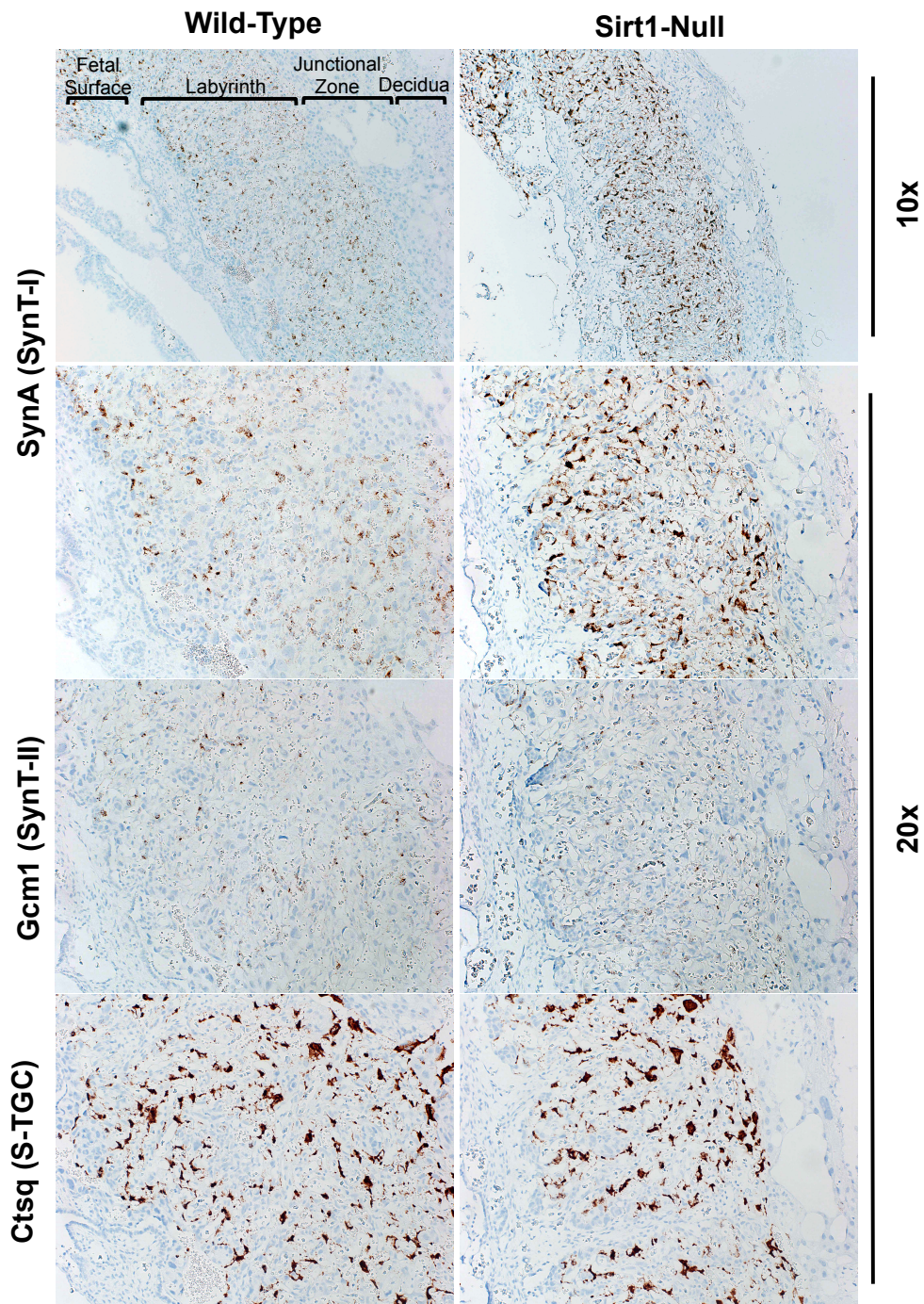


Figure 4.8 Evaluation of labyrinthine layers

In situ hybridization of e13.5 placentas for markers of the three labyrinthine trophoblasts. SynA is expressed in syncytiotrophoblast layer I, Gcm1 in syncytiotrophoblast layer II and Ctsq in sinusoidal TGC. Magnification is 100x (SynA 10x) and 200x (all other images).

expression in the Sirt1-null labyrinth. Conversely, Gcm1 staining appeared to be reduced in null placentas. No difference was detected in Ctsq expression.

4.4 Evaluation of invasive trophoblasts in Sirt1-null placentas

Additional *in situ* hybridization and immunohistochemistry was done to evaluate developmental defects in the junctional zone (Figure 4.9A). The area of Tpbpa positive staining was measured and normalized to the total placental area (determined by pan-cytokeratin staining). The area of Tpbpa-positive staining was statistically significantly reduced in the Sirt1-null placentas compared to WT (Figure 4.9B). Part of the junctional zone is comprised of glycogen trophoblasts. Periodic acid-Schiff (PAS) is a stain used to detect polysaccharides (such as glycogen) and can be used to identify glycogen cells in the placenta. Glycogen cells were identified in both WT and null placentas (Figure 4.10A); however, there was no statistical difference in the area of positive staining when compared to the total placental area (Figure 4.10B).

The spongiotrophoblasts in the junctional zone give rise to invasive trophoblast giant cells. Prl2c2 is a marker of both parietal and spiral artery TGC (Lee et al., 1988). *In situ* hybridization for this marker was performed on both e11.5 and e13.5 WT and null placentas. The null placentas expressed Prl2c2-positive trophoblasts and no differences were detected between Sirt1-null and WT placentas (Figure 4.11). No difference was detected by PL-I *in situ* of e13.5 placentas either (data not shown).

4.5 Progenitor trophoblasts in Sirt1-null placentas

Based on the morphology of the labyrinth and defects in labyrinthine trophoblasts, I hypothesized that in the absence of Sirt1, trophoblasts were trapped in

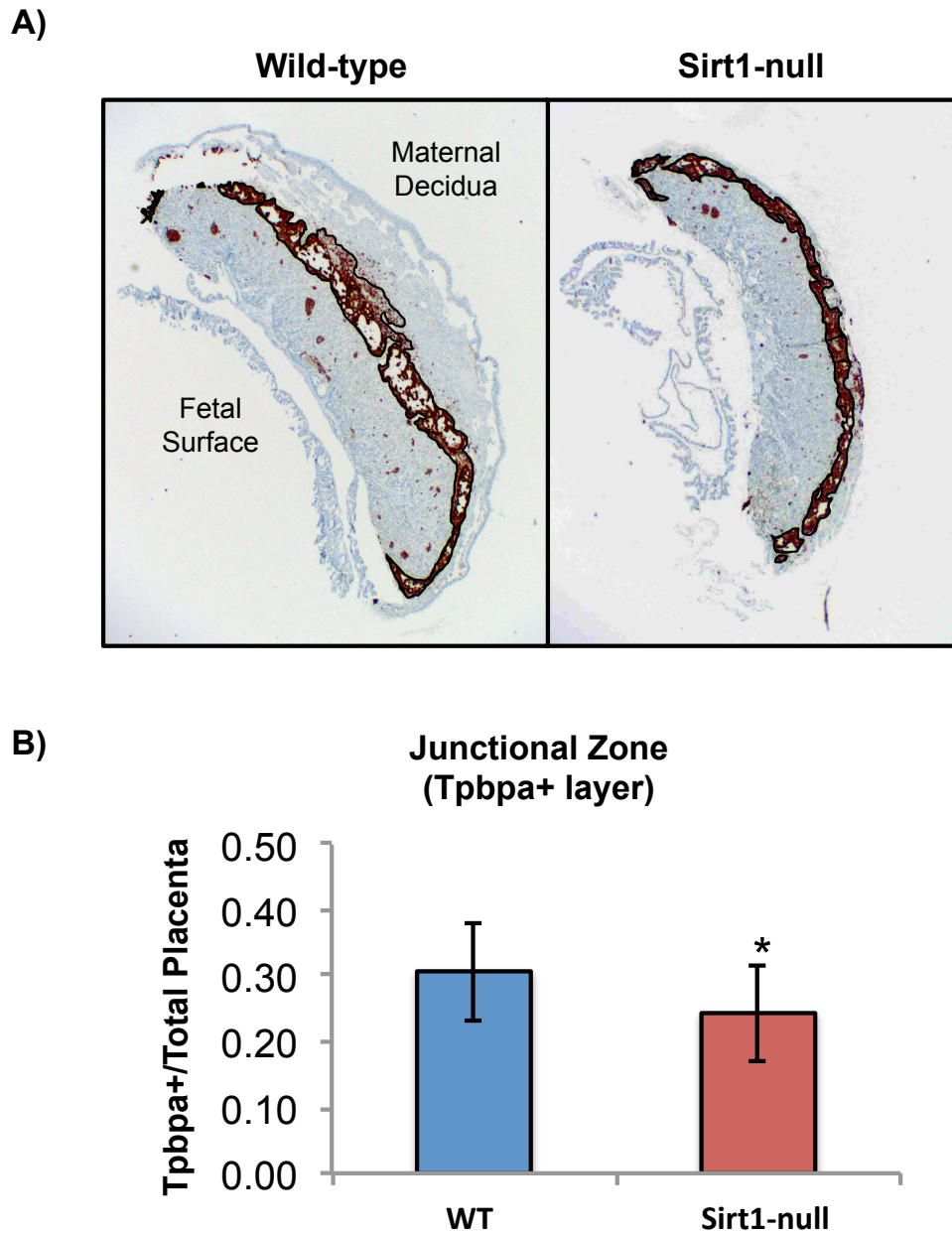


Figure 4.9 Measurement of junctional zone

(A) In situ hybridization for *Tpbpa*, a marker for the junctional zone. Magnification is 20x. (B) *Tpbpa*⁺ area was measured and normalized to total area of the placenta. When compared to WT placentas, the area of the junctional zone was reduced. * $p < 0.05$ by pair-wise t-test.

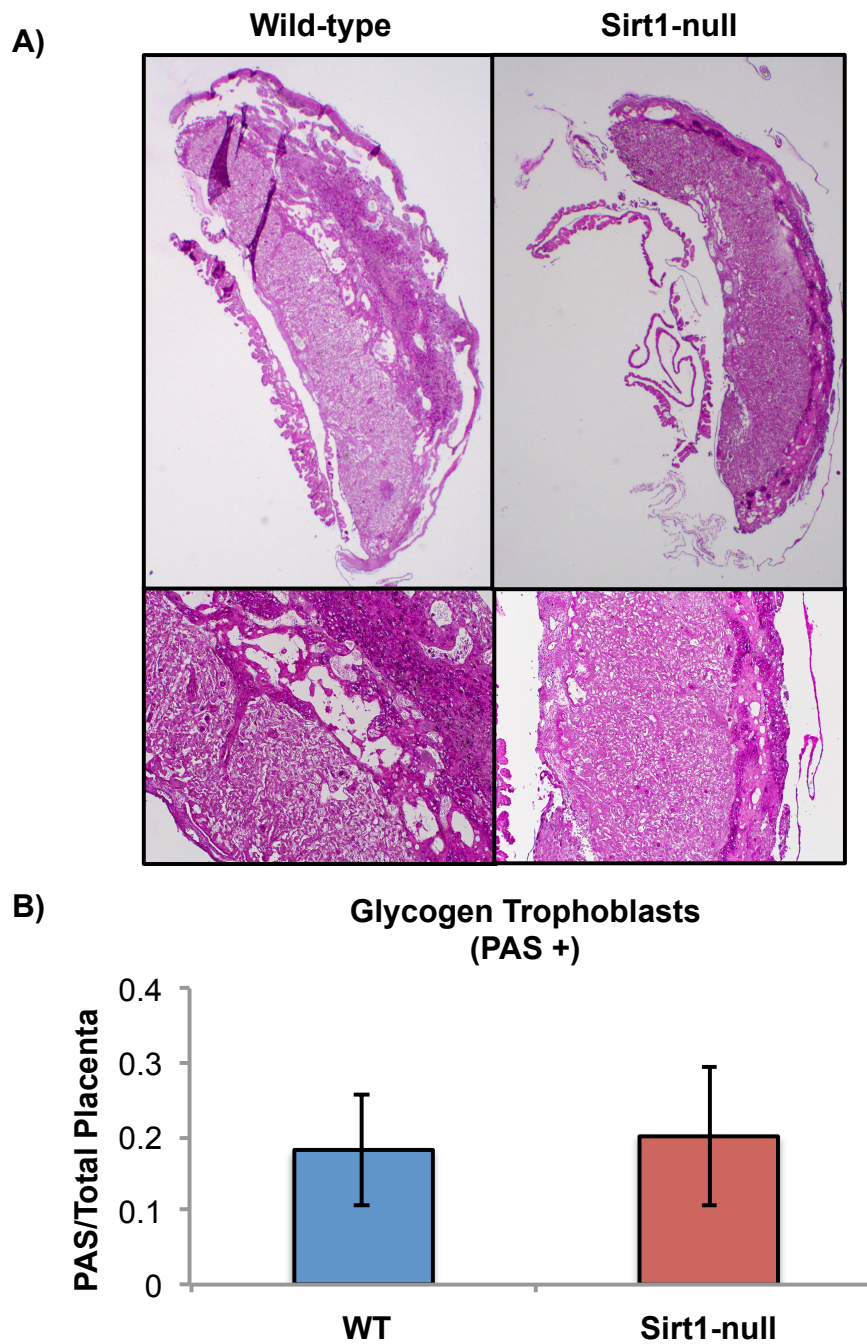


Figure 4.10 PAS stain for glycogen cells

(A) Periodic Acid-Schiff (PAS) staining was done to detect glycogen trophoblasts. Magnification is 20x (top) and 100x (bottom). (B) Measurement of the PAS+ stained area was normalized to total placental area. No difference in area of glycogen cells in WT and null placentas was detected.

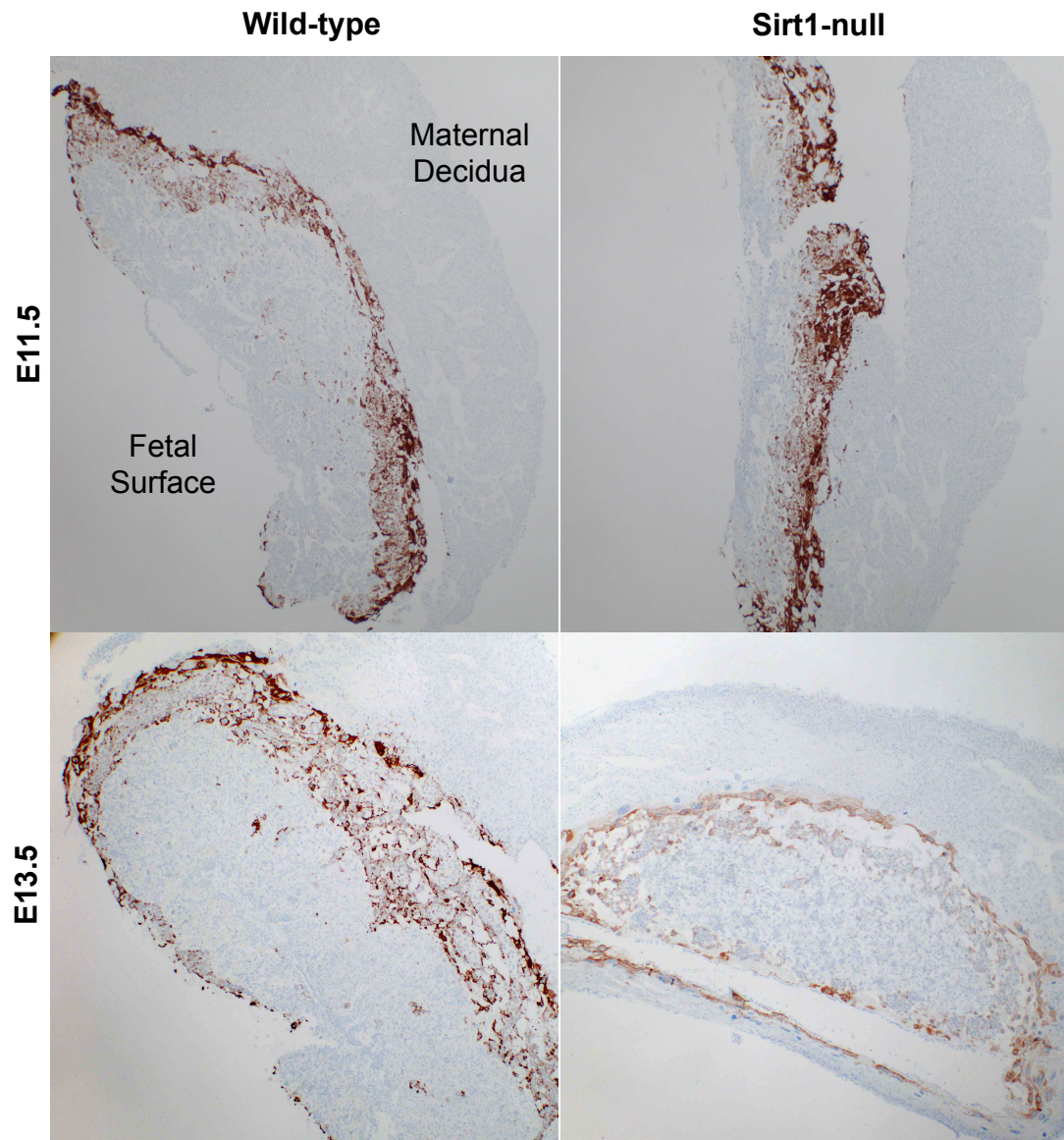


Figure 4.11 *Prlc2* in situ for TGC

In situ hybridization of e11.5 and e13.5 wild-type and Sirt1-null placentas for *Prlc2*, a marker for parietal and spiral artery trophoblast giant cells. There were no differences observed in the expression of this marker. Magnification is 40x.

a progenitor state *in vivo*. Epcam-positive cells have recently been identified as multipotent progenitor cells that give rise to all of the labyrinthine trophoblast lineages (Ueno et al., 2013). In situ hybridization for this marker demonstrated differential expression in the null placentas compared to WT (Figure 4.12). In the WT e13.5 placentas, Epcam was mainly expressed at the edge of the placenta closest to the fetal surface, with a few clusters of Epcam-positive cells in the labyrinth. In the null placentas, there appeared to be more clusters of Epcam-positive cells, with some toward the center of the labyrinth, away from the fetal surface.

In addition to Epcam, I further evaluated other trophoblast progenitor markers by ISH. Rhox4 is a progenitor for SynT-I cells, though no difference was detected in Rhox4-positive trophoblasts in e13.5 placentas (Figure 4.13). Mash2 (Ascl2) is a spongiotrophoblast progenitor marker. Once again, no observable differences were detected in Mash2 in WT and null placentas (Figure 4.14).

4.6 Trophoctoderm-specific knockdown of Sirt1

Upon confirmation of placental defects, I hypothesized that placental defects caused by the lack of placental Sirt1 contribute to the observed growth restriction of Sirt1-null embryos. To test this hypothesis, I aimed to use two different methods to produce placenta-specific knockdown of Sirt1. These methods include using the Cre-Lox system as well as lentivirus infection of blastocysts to produce knockdowns specifically in the trophoctoderm layers. Both of these systems would allow me to observe a wild-type embryo with a null placenta.

Cyp19 is expressed in the placenta so Cyp19-Cre mice can be used for placenta-specific knockout experiments (Wenzel and Leone, 2007). Sirt1-floxed and Cyp19-Cre mice were previously developed mouse lines (Li et al., 2007; Wenzel and

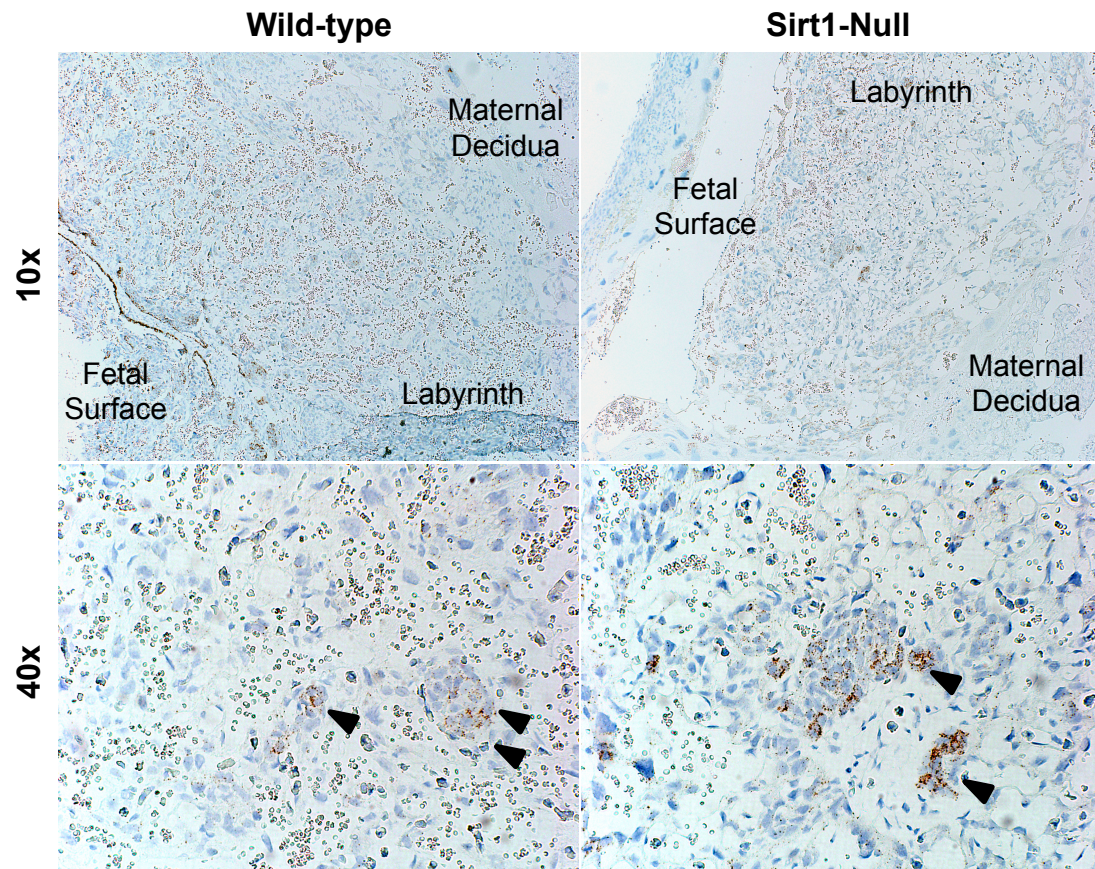


Figure 4.12 Increased Epcam in labyrinth of null placenta

In situ hybridization for Epcam, a labyrinthine lineage progenitor marker. Magnification is 100x (top) and 400x (bottom).

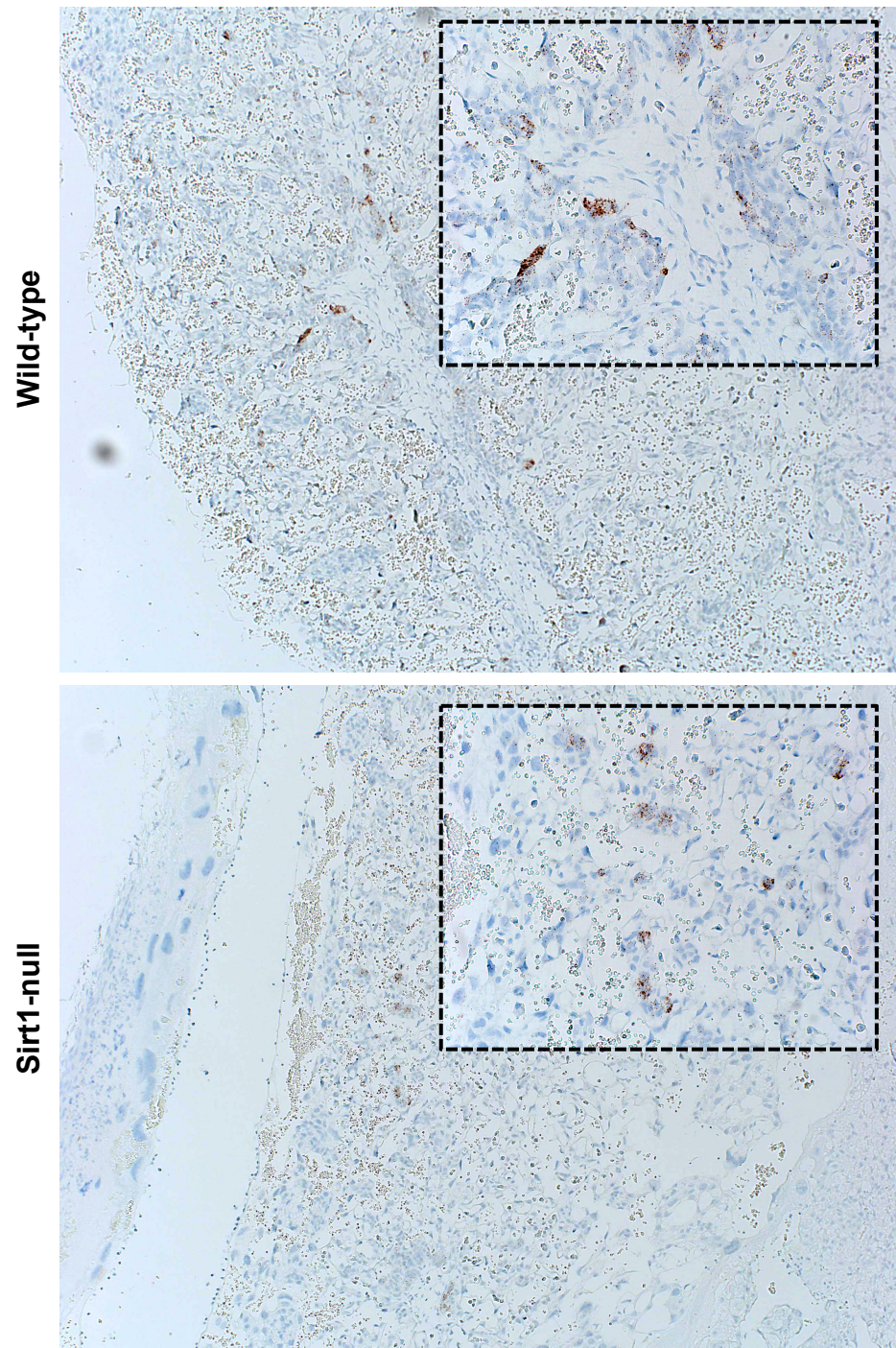


Figure 4.13 Rhox4 is not differentially expressed in null placentas

In situ for Rhox4, an additional labyrinthine progenitor marker, did not detect differences in Rhox4 expression in e13.5 WT and Null placentas. Magnification is 100x and 400x.

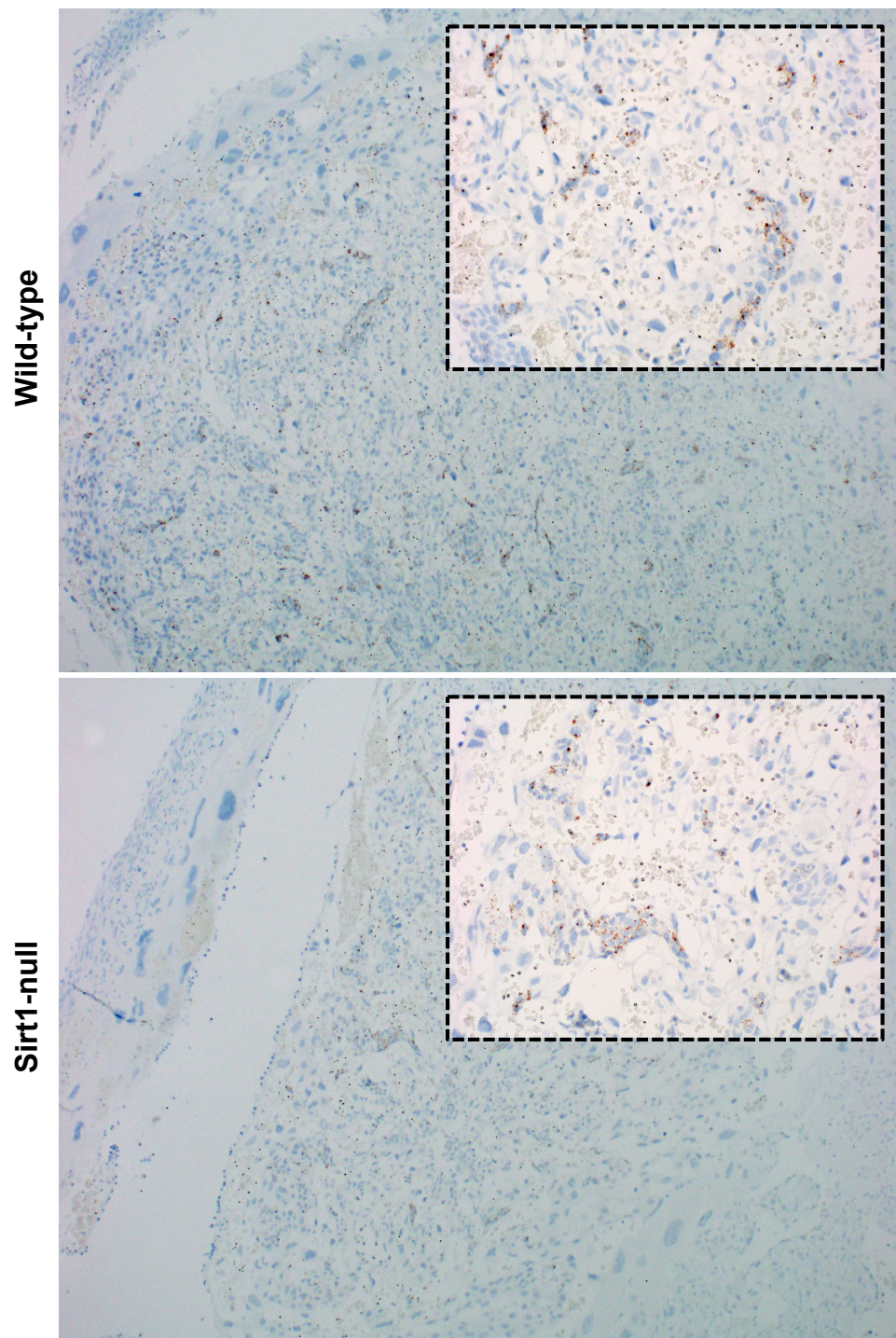


Figure 4.14 In situ hybridization for Mash2

Mash2 in situ hybridization did not demonstrate differential expression of this marker in e13.5 WT and Sirt1-null placentas. Magnification is 100x and 400x.

Leone, 2007). To ultimately produce a null placenta with a wild-type embryo, several breeding experiments were conducted (Figure 4.15). $Sirt1^{flox/flox}$ mice were first crossed to heterozygous $Cyp19^{WT/Cre}$. The resulting pups were all phenotypically WT but produced 50% of each of the two genotypes: $Cyp19^{WT/Cre}Sirt1^{WT/Flox}$ and $Cyp19^{WT/WT}Sirt1^{WT/Flox}$. Mice containing one copy of the Cre allele were then backcrossed to $Sirt1^{Flox/Flox}$ for experimental crosses. These experiments produced mice that were either WT ($Cyp19^{WT/WT}Sirt1^{WT/Flox}$ and $Cyp19^{WT/WT}Sirt1^{Flox/Flox}$), heterozygous placental Sirt1 knockouts ($Cyp19^{WT/Cre}Sirt1^{WT/Flox}$) or homozygous null placentas ($Cyp19^{WT/Cre}Sirt1^{Flox/Flox}$). These embryos and placentas are currently being analyzed.

In addition to using the Cre-Lox system, I am also using lentivirus to knockdown placental Sirt1 via a secondary *in vivo* method (Figure 4.16). Lentivirus knockdown and overexpression experiments have been done by other labs and have shown successful, placental specific expression of genes of interest (Georgiades et al., 2007; Okada et al., 2007; Kaufman et al., 2014). To establish this technique at UCSD, I worked with the Transgenic Mouse Core Facility to optimize these experiments. I routinely make lentivirus at high titer. Flushed e2.5 blastocysts were then infected with lentivirus, and then reintroduced into a pseudopregnant female mouse. Evaluation of these placentas and embryos at later timepoints should display placenta specific expression of constructs. Optimization experiments were done using CMV-GFP lentivirus, produced in lab. Thus far, we have managed to get very weak expression of a few trophoblasts single cells in the mouse placenta at e13.5 (Figure 4.17).

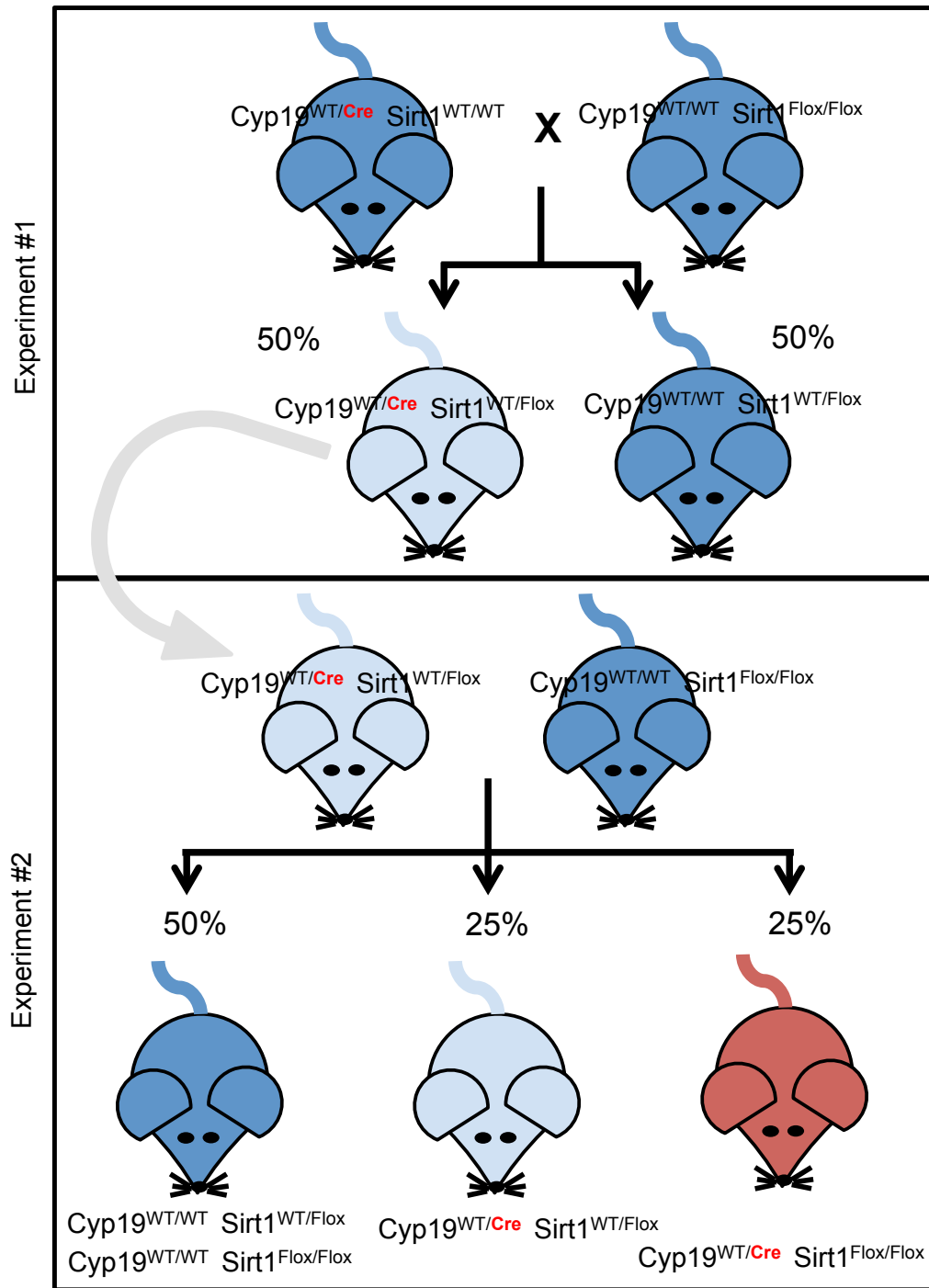


Figure 4.15 Placenta-specific Sirt1 knockdown using Cre-Lox

Schematic illustrating the matings performed between $\text{Sirt1}^{\text{Flox/Flox}}$ and $\text{Cyp19}^{\text{WT/Cre}}$ mice to produce a Sirt1-null placenta and WT embryo.

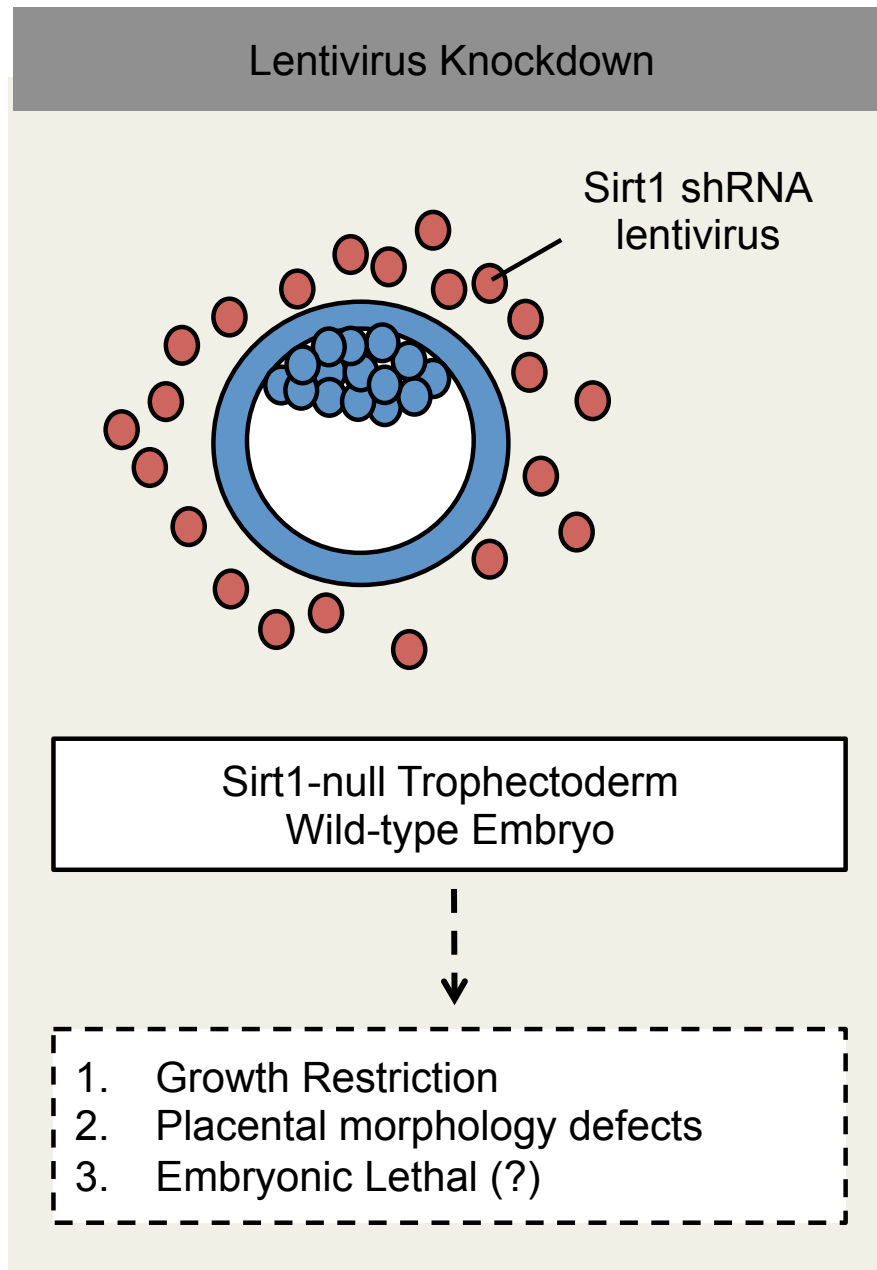


Figure 4.16 Placenta-specific Sirt1 knockdown using lentivirus

Diagram describing the use of lentivirus to produce a placenta-specific knockdown of Sirt1. Blastocysts were infected with lentivirus and should produce a Sirt1-null placenta and a WT embryo.

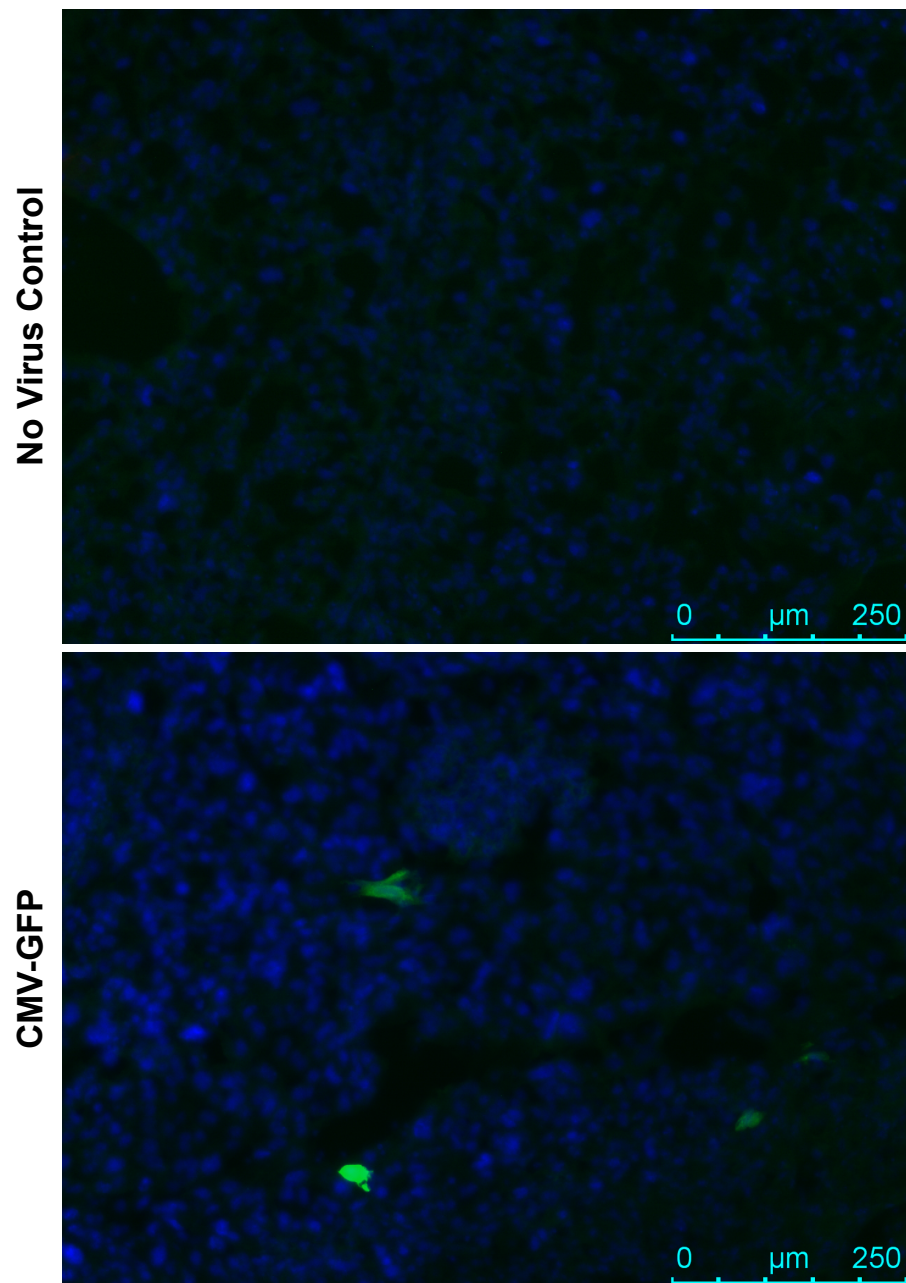


Figure 4.17 Optimization of placenta-specific knockdown using CMV-GFP lentivirus

CMV-GFP lentivirus was used to infect blastocysts to test for placenta -specific expression of constructs using this method. Placentas were counterstained with DAPI. Green cells observed are fluorescence from GFP+ cells in the placenta.

Chapter 4, in part, contains material that is currently being prepared for submission for publication of the material. Arul Nambi Rajan Kanaga, Soncin Francesca, Moretto-Zita Matteo, Iyer Pooja, Tache Veronique, Pizzo Donald, McBurney Michael, Parast Mana M. The dissertation author was the primary investigator and author of this material.

Chapter 5

Characterization of the
Differentiation Related Pathways
Downstream of Sirt1 in
Trophoblasts

5.1 TGF β Signaling in WT and Sirt1-null trophoblasts

One of the many signaling cascades important for placental development is the TGF β superfamily. This family includes Activin, Nodal, TGF β and BMP signaling pathways. TGF β and Activin are necessary, with FGF4, to maintain trophoblast stem cells (Erlebacher et al., 2004). TS cells also produce BMP4, another member of this signaling superfamily. BMP signaling is not only important for embryonic growth (Murohashi et al., 2010) but preliminary data from our lab (unpublished) suggests it may play a role in trophoblast stemness as well. Based on the above, I asked if the Sirt1-null differentiation defect resulted from dysregulation of TGF β signaling.

First, I evaluated Activin signaling, given the importance of Activin A in maintaining TS cells. Wild-type and Sirt1-null TS cells were serum starved for 5 hours to remove all residual Activin signaling, after which 10ng/ml Activin A was re-introduced. The level of Activin signaling was determined by examination of phosphorylated Smad2/3 (Figure 5.1). Upon 1 hour of Activin A treatment in minimal media, WT cells exhibited an increase in p-Smad 2/3 which decreased slightly after 3 hours. Though Sirt1-null TS cells demonstrated a similar pattern of p-Smad 2/3, the level of expression was noticeably reduced. Total Smad2 and Smad3 were also slightly less in the null cells.

Since the Activin/TGF β /Nodal branch of TGF β signaling family is reduced in the Sirt1-null cells, I hypothesized that BMP signaling may also be altered. I therefore measured phosphorylated Smad 1/5/8 expression to determine BMP signaling. In WT cells differentiated for 48 hours, p-Smad 1/5/8 did not decrease until around 48 hours; however, in the Sirt1-null TS cells it was almost entirely undetectable from 0 to 48 hours (Figure 5.2A). In addition, while total Smad1 protein levels did not change

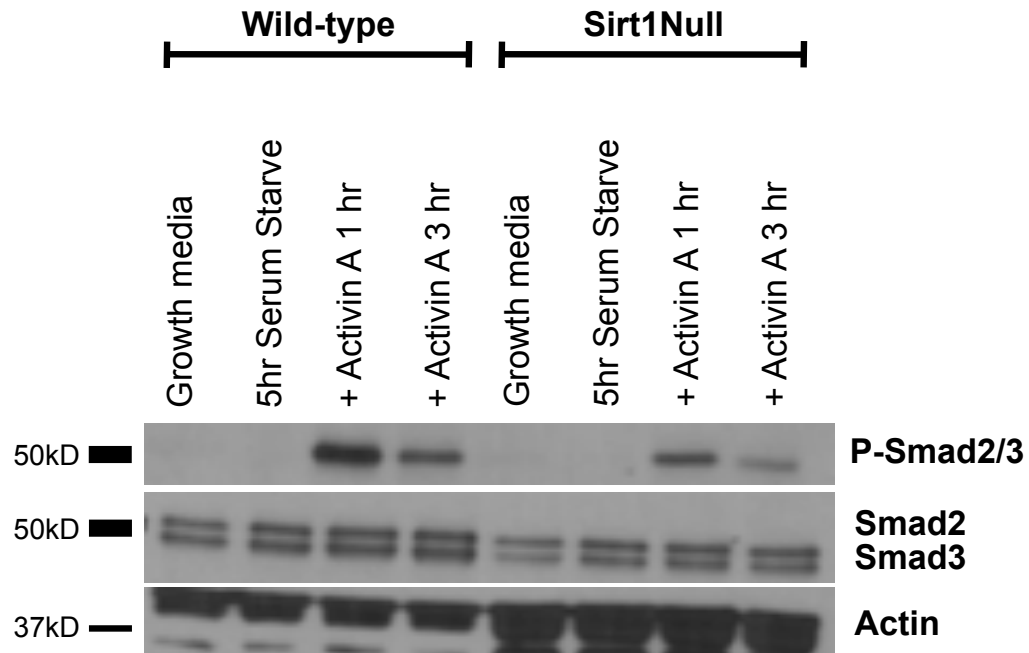


Figure 5.1 Activin signaling reduced in Sirt1-null trophoblasts

Western blot of phosphorylated-Smad2/3, total Smad2/3 and β -Actin. Wild-type and Sirt1-null TS cells were serum starved for 5 hours in serum-free TS media. Cells were then treated with 10ng/ml ActivinA in serum-free media and collected after 1 and 3 hours of treatment.

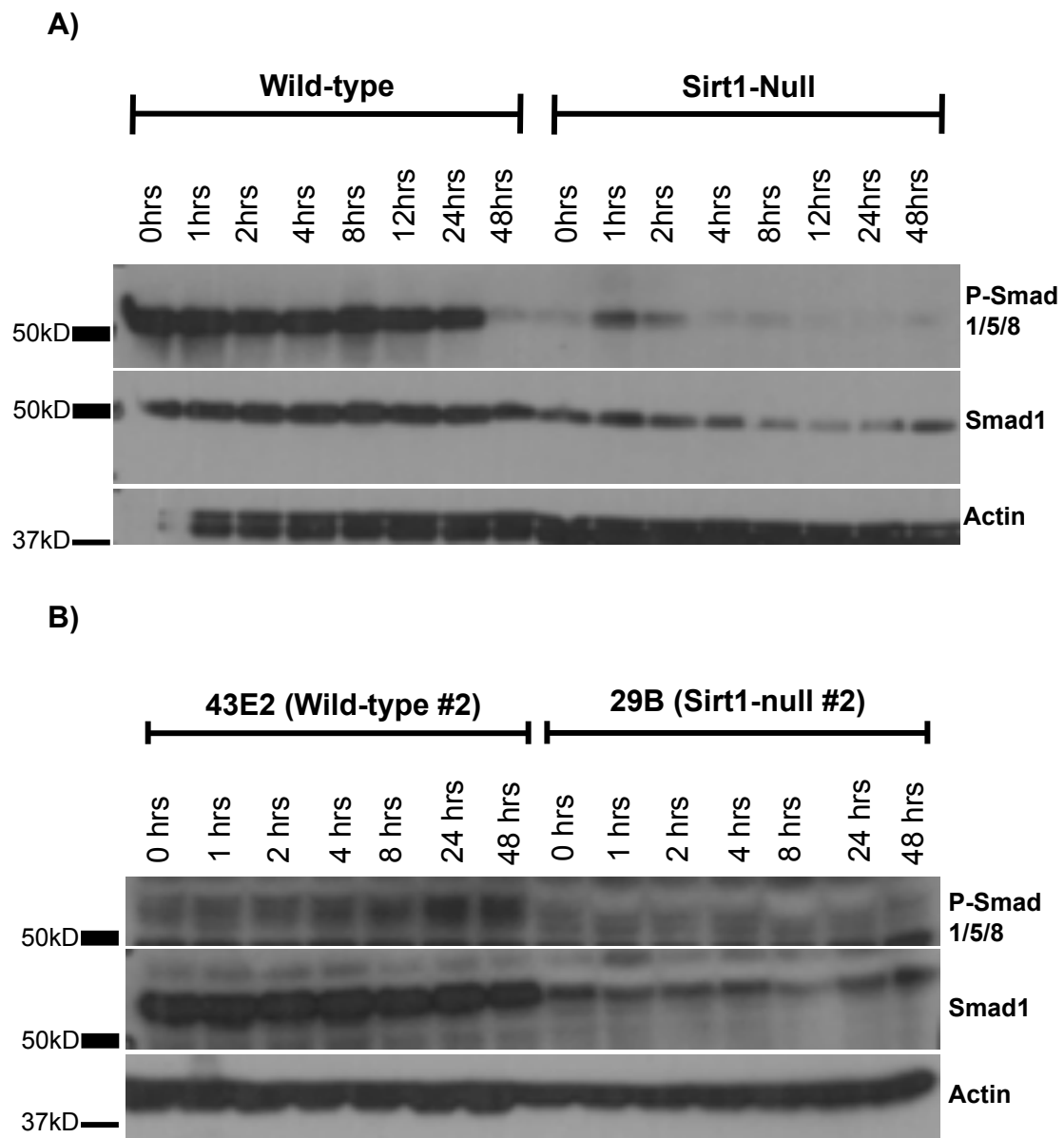


Figure 5.2 BMP signaling via Smad 1/5/8 reduced in trophoblasts lacking Sirt1

(A) Western blot of phosphorylated Smad1/5/8, Smad1 and β -Actin in wild-type and Sirt1-null TS cells differentiated for 48 hours in standard differentiation media. (B) Experiment was repeated in secondary wild-type #2 (43E2) and Sirt1-null #2 (29B) TS cell lines.

during WT differentiation, Smad1 expression was consistently reduced in the null trophoblasts. This expression pattern of reduced p-Smad1/5/8 and Smad1 protein was also observed in a second WT and Sirt1-null cell line (Figure 5.2B).

Next, I wanted to know, if in the absence of Sirt1, TS cells had the appropriate receptors to respond to BMP signaling. qRT-PCR for BMP receptors showed that null trophoblasts did express BMP-R1A, BMP-R1B (Figure 5.3A) and BMP-R2 (Figure 5.3B). Expression of RNA of the BMP4 ligand was reduced in the null TS cells and was interestingly slightly higher during trophoblast differentiation d1 in the absence of Sirt1 (Figure 5.3C).

Given the reduced BMP signaling and reduced p-Smad1/5/8 in null TS cells, I hypothesized that treatment of null TS cells with BMP4 may increase p-Smad1/5/8 levels and, upon treatment of differentiation conditions, would partially rescue the null trophoblast phenotype, and demonstrate an increase in trophoblast differentiation markers. WT and null TS cells maintained in growth conditions supplemented with 100ng/ml of BMP4 did exhibit an increase in p-Smad1/5/8 (Figure 5.4). However, despite the increase in p-Smad 1/5/8, the null cells continued to exhibit blunted differentiation into terminal trophoblast lineage markers such as SynA, Tpbpa and Prl3b1 (Figure 5.5). Expressions of TS markers were not evaluated.

5.2 HGF signaling in WT and Sirt1-null trophoblasts

HGF signaling is necessary for the maintenance of the Epcam-positive labyrinthine cells (Ueno et al., 2013). I hypothesized that HGF signaling is maintained in differentiated null TS cells and halts these TS cells in an Epcam+ progenitor stage. In WT TS cells, Epcam protein is highest on WT d0 and decreases with differentiation up to d3 and is undetectable thereafter (Figure 5.6A). This expression corresponds to

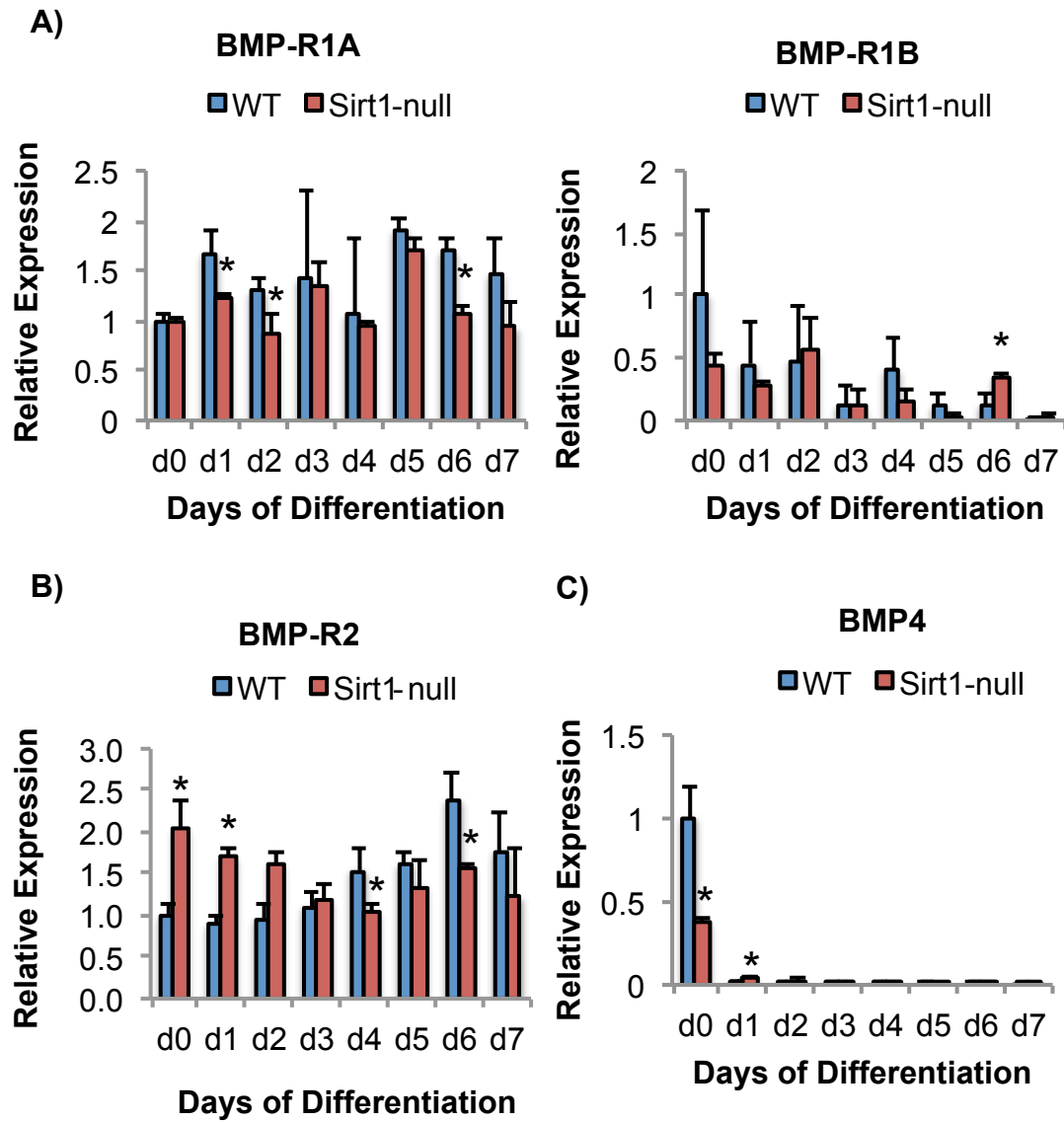


Figure 5.3 RNA expression of BMP4 and BMP receptors

qRT-PCR for BMP receptor 1A and 1B (A), BMP receptor 2 (B) and BMP4 (C). Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

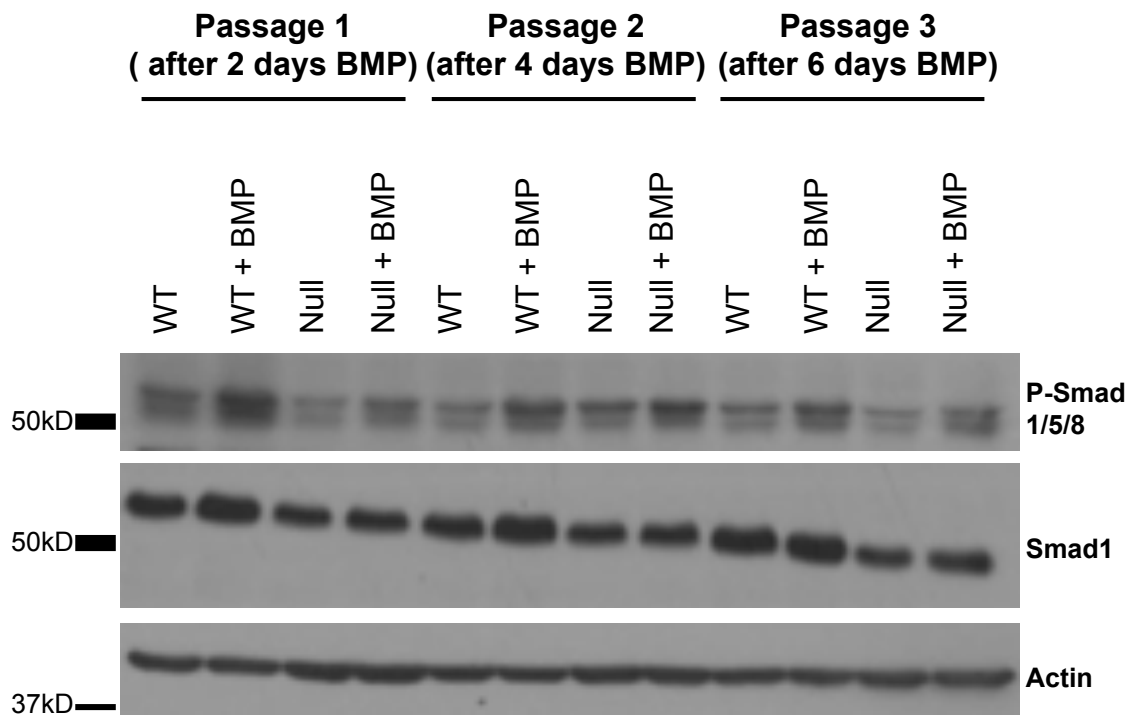


Figure 5.4 Maintenance of WT and Null TS cells in presence of BMP4

Western blot of phosphorylated Smad1/5/8, Smad1 and β -Actin in wild-type and Sirt1-null TS cells. TS cells were maintained in either TS growth media or growth media supplemented with 100ng/ml BMP4. Both WT and Null cells treated with BMP4 demonstrated an increase in phosphorylated Smad1/5/8.

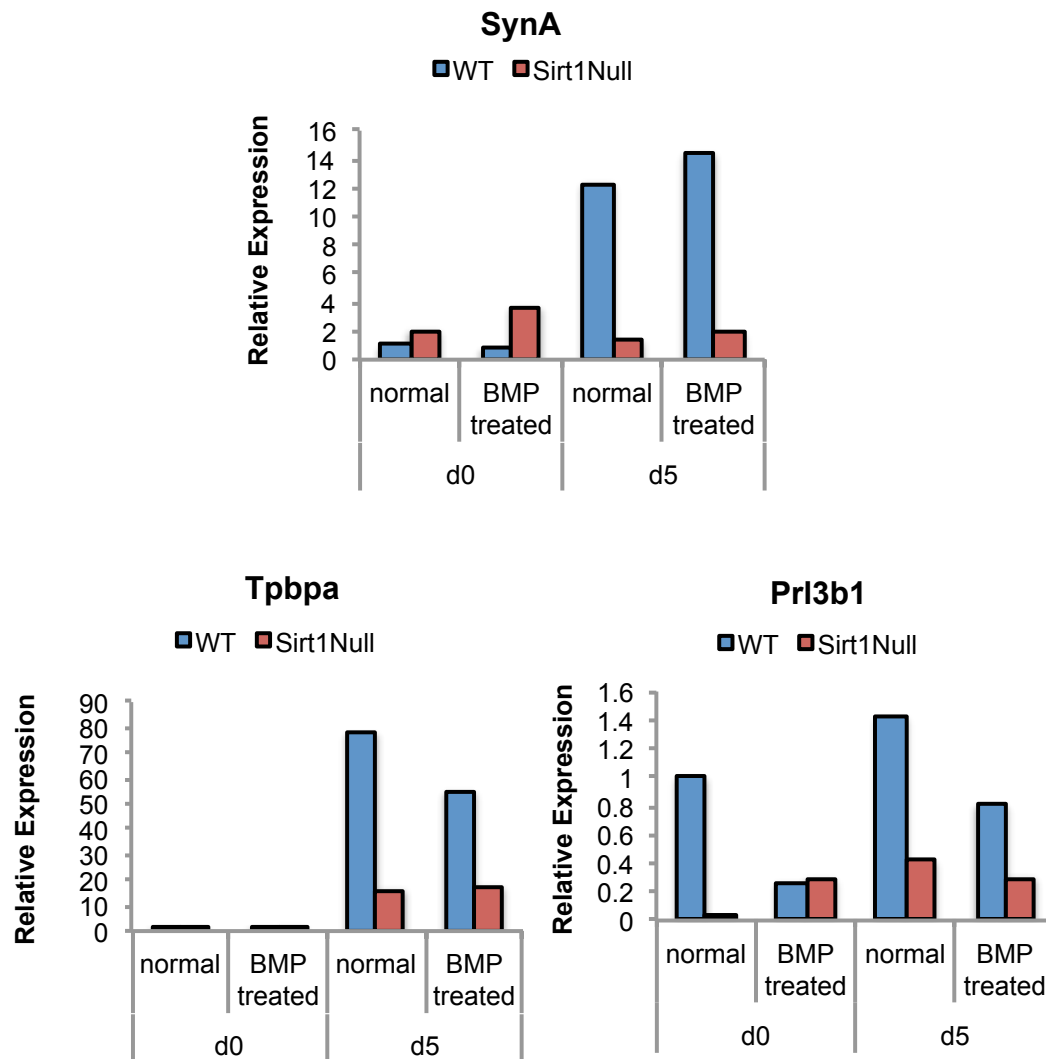


Figure 5.5 BMP treatment fails to rescue Sirt1-null trophoblast differentiation defect

qRT-PCR for trophoblast lineage markers after TS cells were maintained in growth media supplemented with 100ng/ml BMP4. Values adjusted to WT d0 and normalized to 18S ribosomal RNA.

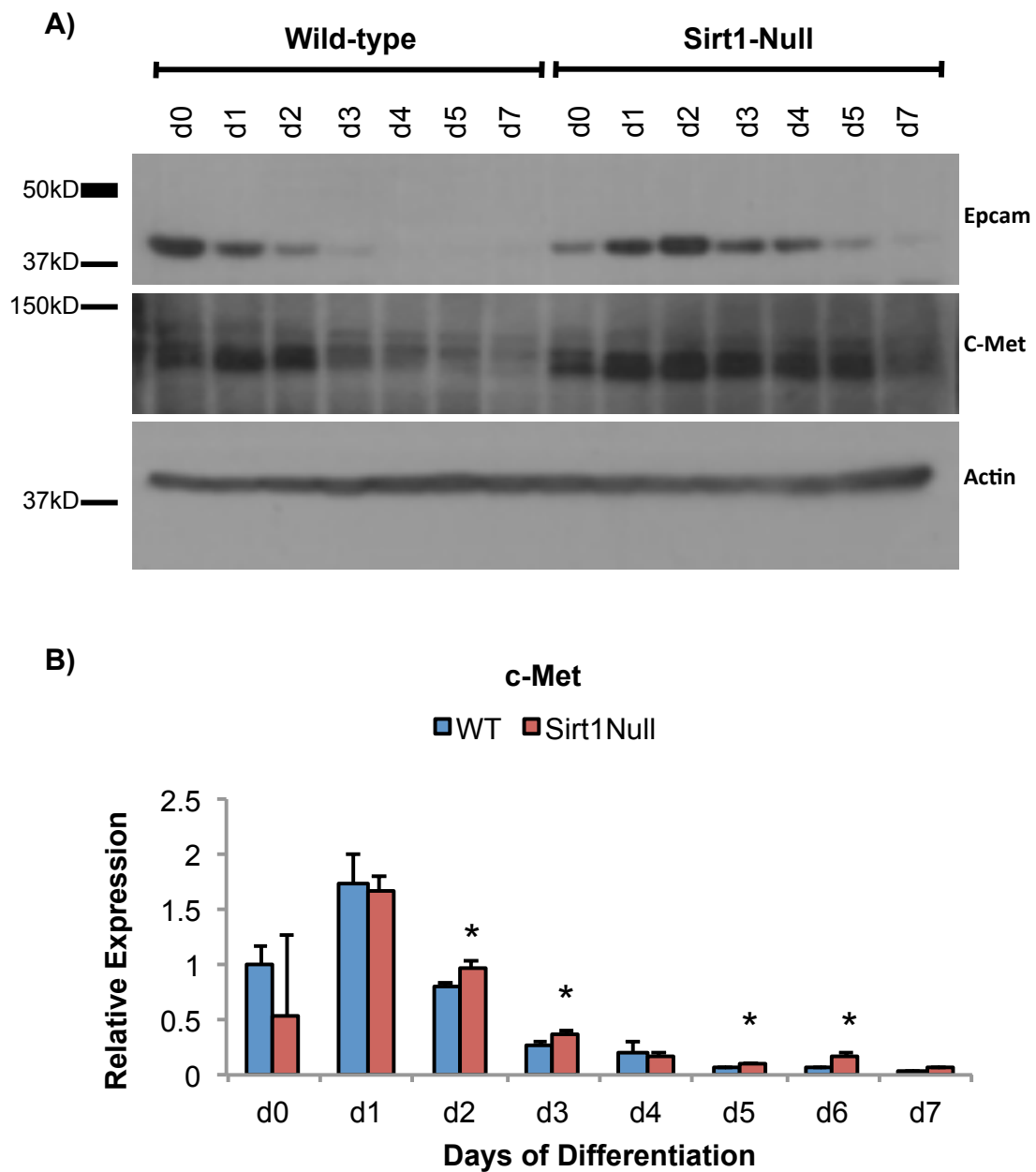


Figure 5.6 c-Met and Epcam protein are maintained during Sirt1-null differentiation

(A) Western blot of Epcam, c-Met and β -Actin of cells in standard differentiation media. (B) qRT-PCR of c-Met RNA expression during differentiation. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

an increase and peak of the HGF receptor, c-Met, on d1-d2 and subsequent decrease on d3 of differentiation. However, in the absence of Sirt1, null trophoblasts exhibit an increase and peak of Epcam on d2, after which the protein decreases but is detectable up until differentiation d7. Similarly, c-Met increases on d1 and is maintained at higher levels compared to WT until the end of differentiation. Interestingly, this increase in c-Met expression was not detected at the RNA level (Figure 5.6B).

To test if HGF signaling, not just c-Met expression, is dysregulated I next examined phosphorylated c-Met expression. I serum starved WT TS cells for 5 hours and then treated cells with increasing concentrations of HGF and identified 25ng/ml to be the optimal concentration to detect p-c-Met (Figure 5.7). Next, I treated differentiated trophoblasts with HGF to determine if, in the absence of Sirt1, TS cells exhibited an increased response to HGF. I performed a standard differentiation timecourse of WT and null TS cells but prior to collection, cells were serum starved for 5 hours in minimal media and then treated with 25ng/ml HGF for 15 minutes prior to collection for analysis. c-Met has three known phosphorylation sites. Phosphorylation at tyr1234/1235 and tyr1349 indicate HGF binding and homodimerization while tyr1003 phosphorylation indicates negative regulation and degradation of the receptor (Organ and Tsao, 2011). No differences were detected in phosphorylated c-Met tyr1234/1235 or tyr1349 (Figure 5.8). There is however a slight reduction in p-c-Met tyr1003 in the Sirt1-null trophoblasts.

In the absence of Sirt1, TS cells exhibit an increase in Epcam expression at both the RNA and protein levels and reduced terminal differentiation markers. I therefore hypothesized that WT TS cells differentiated in the presence of HGF-cMet

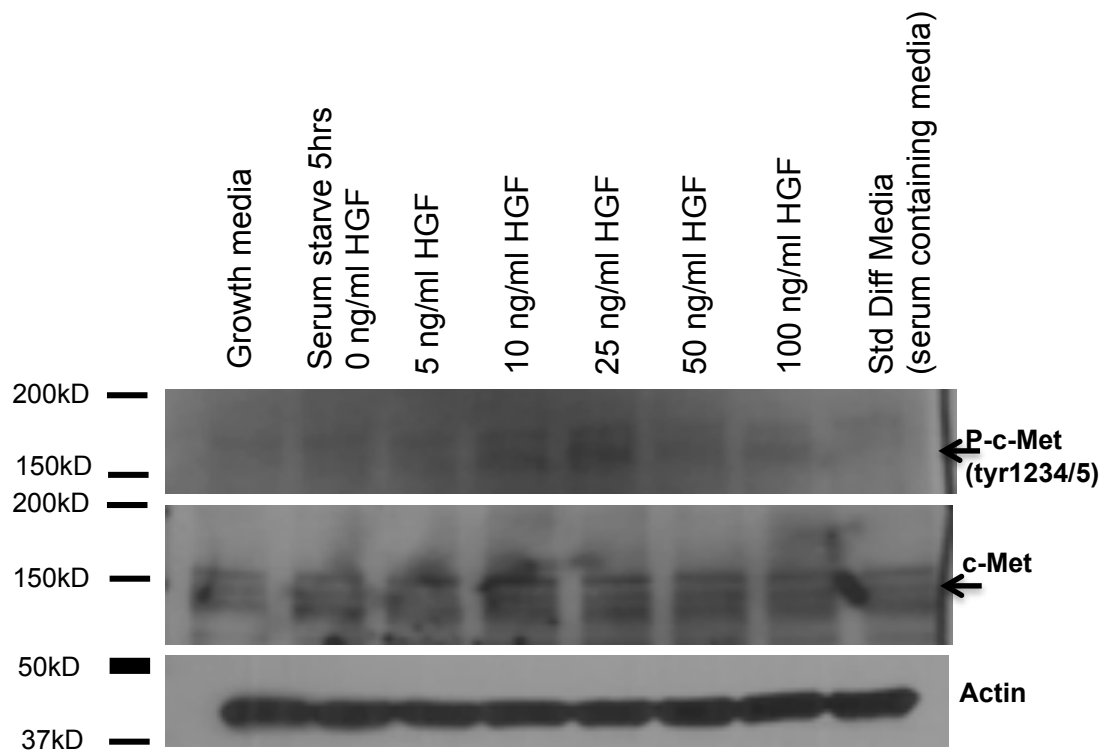


Figure 5.7 HGF dose-response treatment

Western blot of phosphorylated c-Met (tyr1234/1235), c-Met and β -Actin of wild-type cells serum-starved for 5 hours and then treated with 0 – 100ng/ml of HGF for 15 minutes.

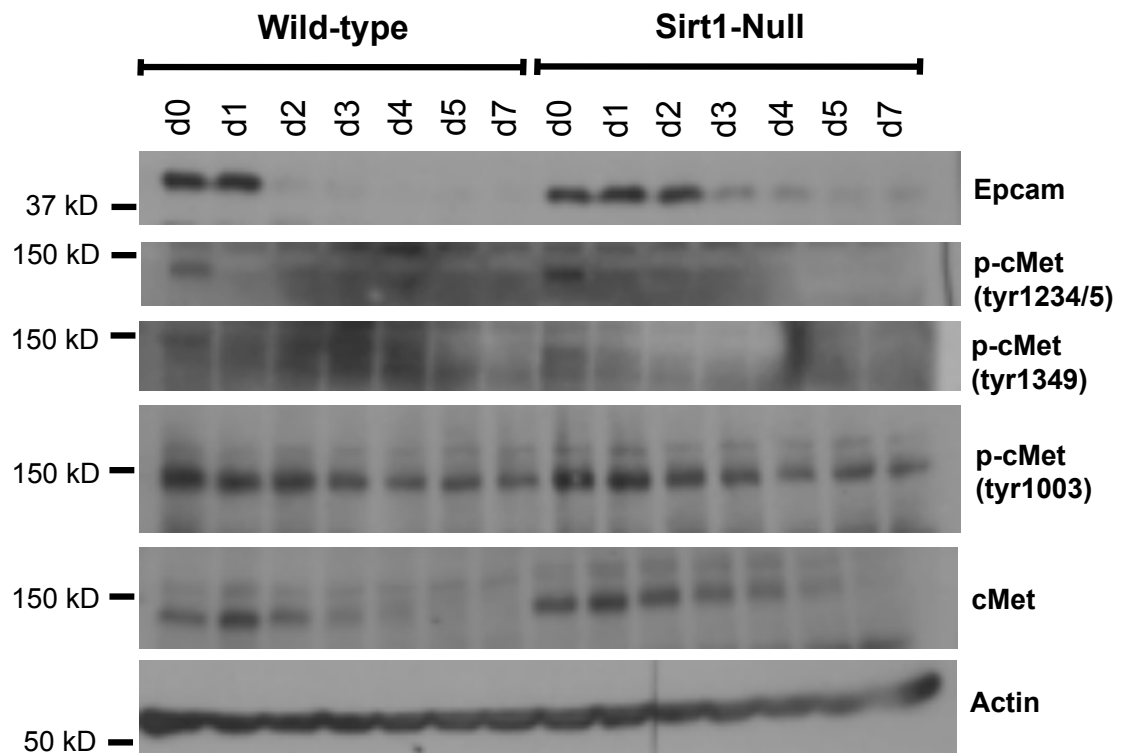


Figure 5.8 HGF/c-Met signaling is not different during wild-type and Sirt1-null trophoblast differentiation

Western blot of phosphorylated c-Met at tyr1234/1235, tyr1249 and tyr1003 in addition to c-Met and β -Actin. Wild-type and Sirt1-null cells were differentiated in standard differentiation media. 3 hours prior to collection, cells were serum starved and then pulsed with 25ng/ml HGF.

signaling would recapitulate the null differentiation phenotype. To test this, WT and null TS cells were treated with differentiation media supplemented with 25ng/ml HGF for 5 days. WT cells treated with HGF did differentiate but did not exhibit a statistically significant decrease in SynA compared to normally differentiated trophoblasts (Figure 5.9A). Treated cells did demonstrate a statistically significant decrease in Tpbpa and Prl3b1, although not down to the levels of Sirt1-null cells; reduced expression was also observed in null TS cells, though not statistically significant for Prl3b1 (Figure 5.9B). However, no increase in Epcam was observed in the differentiated WT cells at either the protein (Figure 5.10A) or RNA (Figure 5.10B) levels.

5.3 Assessment of signaling cascades downstream of c-Met

A variety of signaling pathways are activated downstream of the c-Met receptor. These include MAPK, ERK, JNKs, PI3K-AKT and STAT pathways, among others (Trusolino et al., 2010; Organ and Tsao, 2011). Of these, AKT and MAPK signaling have been implicated in maintenance of neural progenitors. Specifically, maintenance of these signaling pathways through phosphorylated AKT and p38 MAPK after Sirt1 inactivation results in overproduction of oligodendrocyte progenitor cells (Rafalski et al., 2013). Furthermore, p38MAPK-null placentas exhibit placental defects including loss of labyrinthine layer, reduced spongiotrophoblasts and poor placental vasculature (Mudgett et al., 2000). AKT signaling has not been well studied in mouse placentas but is implicated in human cell models to promote extravillous trophoblast migration (Cartwright et al., 2002; Qiu et al., 2004).

Thus, I evaluated expression of phosphorylated p38 MAPK and AKT during trophoblast differentiation. Examining early differentiation time-points from 0 to 48 hours, p-p38 MAPK expression did not change with differentiation nor was signaling

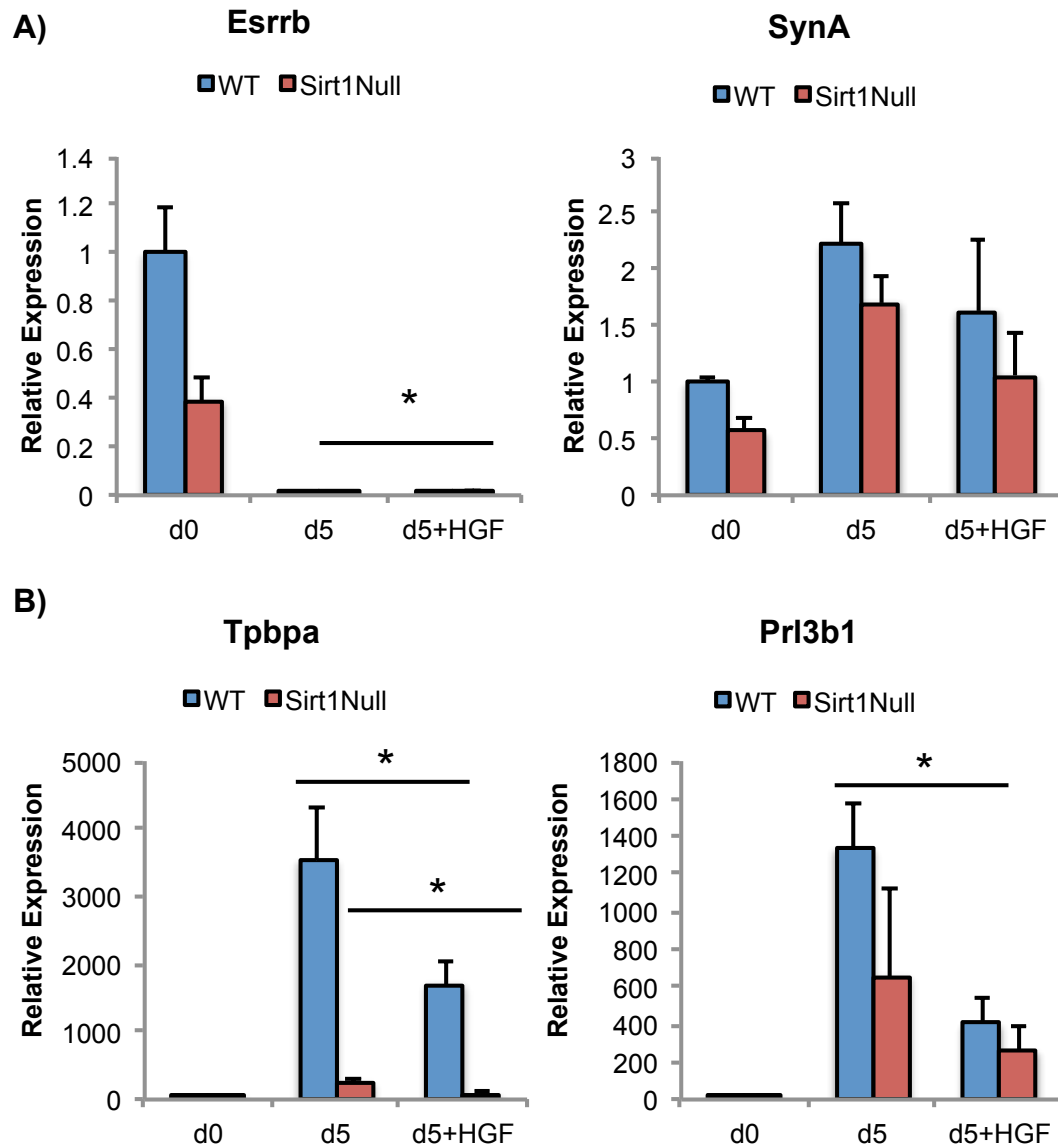
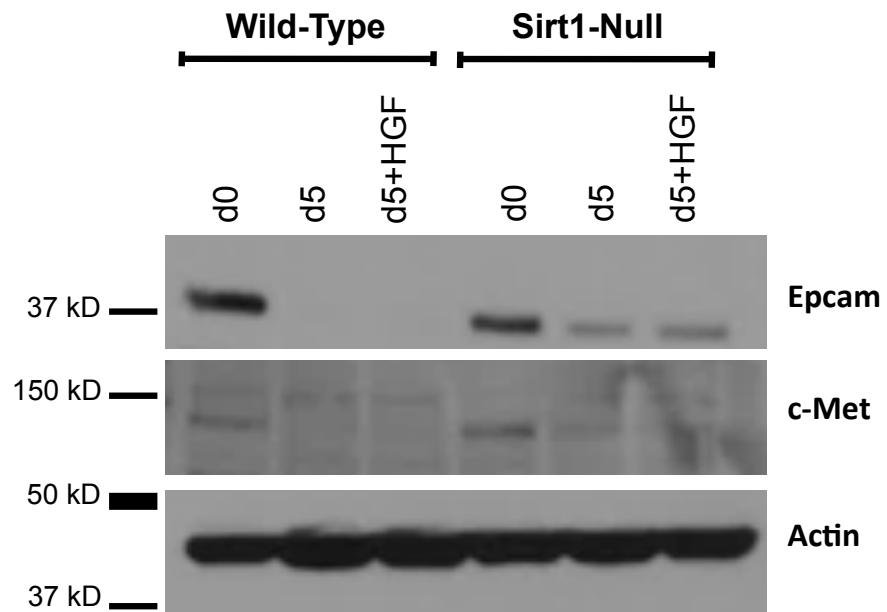


Figure 5.9 Differentiation of Wild-type and Sirt1-null TS cells in presence of HGF

Wild-type and Sirt1-null TS cells were differentiated for 5 days with or without 25ng/ml HGF. qRT-PCR was used to evaluate differentiation markers of TS cells and labyrinthine trophoblasts (A) as well as invasive trophoblasts including Tpbpa (spongiotrophoblasts) and Prl3b1 (TGC) (B). Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

A)



B)

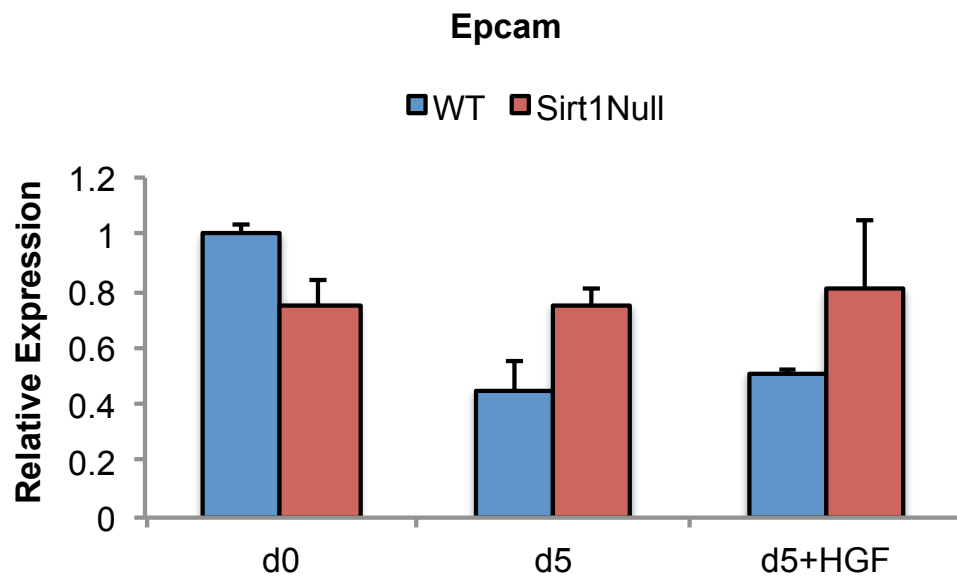


Figure 5.10 HGF does not maintain Epcam and c-Met in WT trophoblasts

(A) Western blot of Epcam, c-Met and β -Actin of WT and Sirt1-null TS cells differentiated for 5 days with or without 25ng/ml HGF. (B) qRT-PCR of Epcam RNA expression.

altered in the null cells compared to WT, despite a slight decrease in p38/MAPK protein in the null cells (Figure 5.11A). I also examined p-p38/MAPK expression at later differentiation timepoints from day 0 through 8; however, no differences in signaling were detected, although again, the total p38/MAPK in the null cells was slightly reduced (Figure 5.11B). Detection of total and phosphorylated AKT during early differentiation timepoints also was not different in WT and Sirt1-null trophoblasts (Figure 5.12).

5.4 Assessment of signaling pathways important in trophoblast and placental development

There are many signaling cascades important in trophoblast differentiation and placental development. Sirt1 also regulates some of these pathways in other cellular models. I therefore hypothesized that Sirt1 may modulate pathways such as Wnt and Notch signaling in trophoblasts. Canonical Wnt is important for Gcm1 expression and chorioallantoic attachment (Lu et al., 2013; Soncin et al., 2015). In relation to Sirt1, it has been shown that β -catenin, a transcription factor in the Wnt pathway can be regulated by Sirt1. In mesenchymal stem cells (MSCs), Sirt1 deacetylates β -catenin and facilitates its localization to the nucleus (Simic et al., 2013b). In MSCs lacking Sirt1, β -catenin fails to localize to the nucleus, resulting in defects in osteoblast differentiation. I tested to see if in the absence of placental Sirt1, β -catenin exhibits a similar defect in nuclear localization. Immunofluorescence staining of the original WT and null TS cell lines demonstrated that in both undifferentiated and differentiated WT trophoblasts, β -catenin localized to the cytoplasm and that Sirt1-null cells exhibit a similar expression pattern (Figure 5.13).

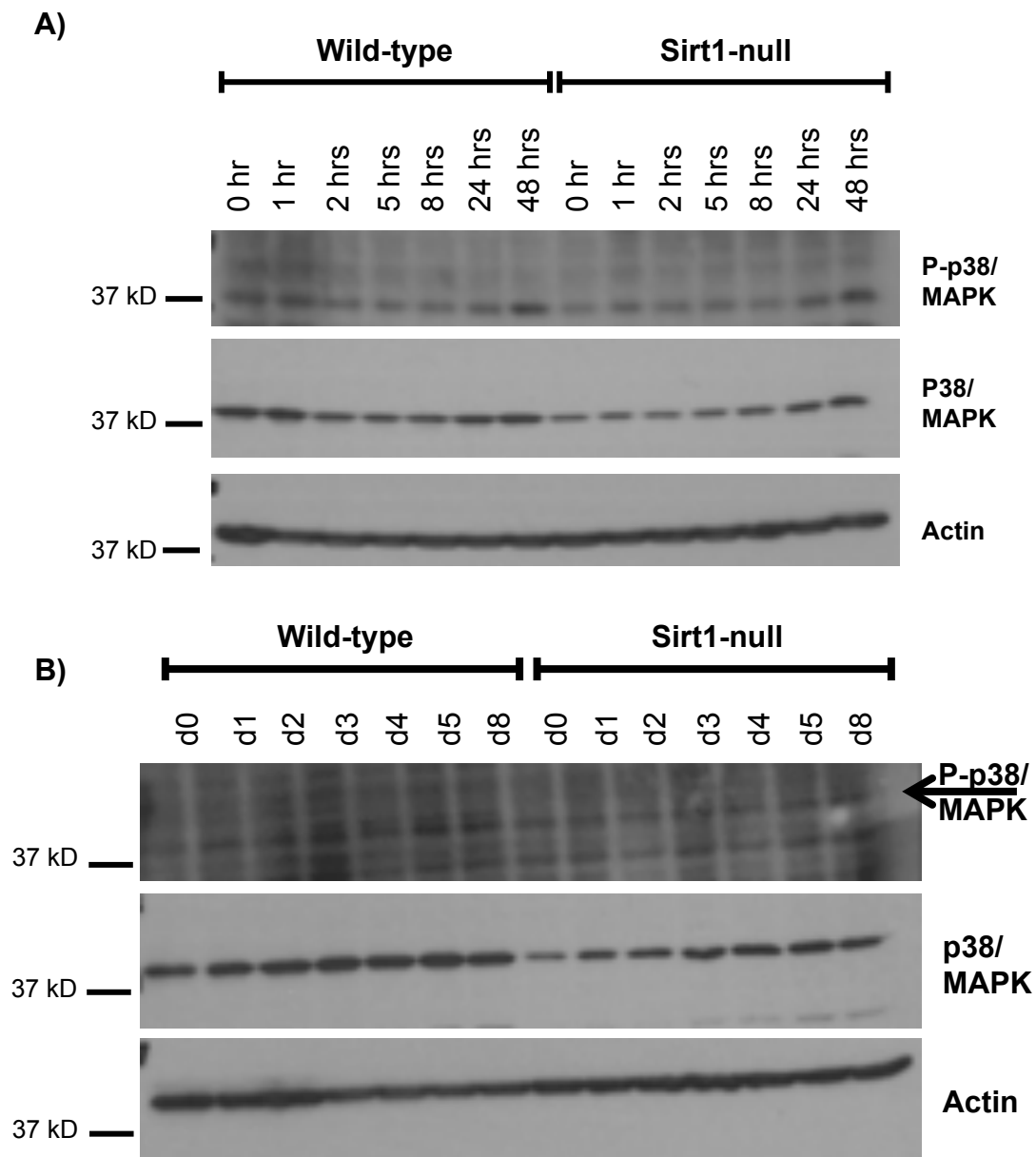


Figure 5.11 p38/MAPK signaling is not altered in trophoblasts lacking Sirt1
 Western blot of phosphorylated and total p38/MAPK in addition to β -Actin in wild-type and Sirt1-null TS cells differentiated under standard differentiation conditions within first 48 hours of differentiation (A) and during 7 days of differentiation (B).

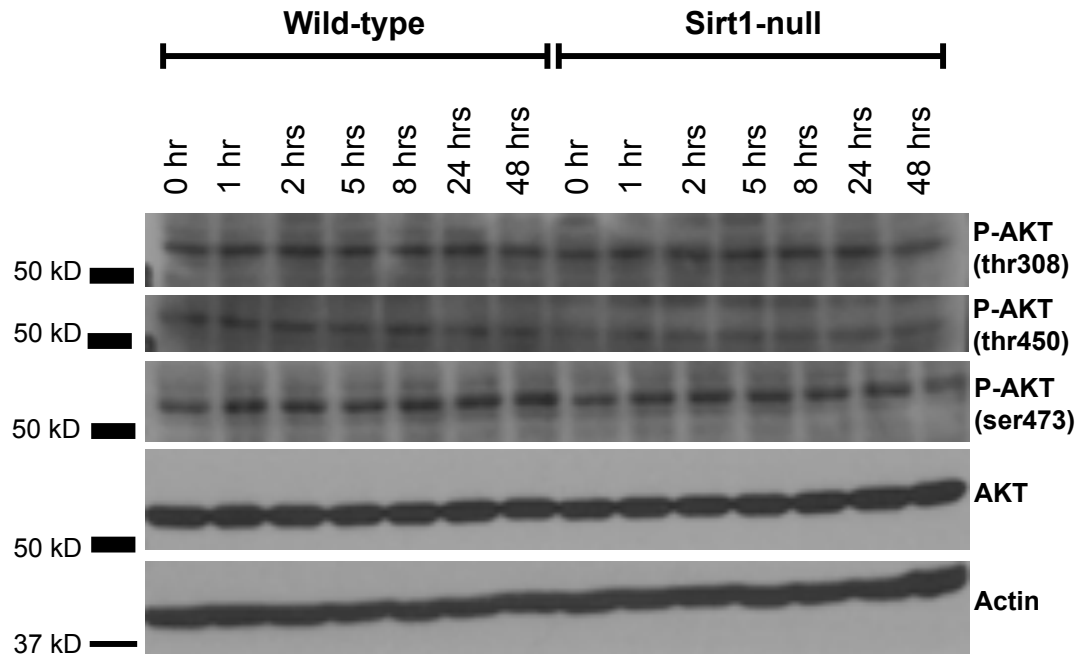


Figure 5.12 AKT signaling is not altered during wild-type and Sirt1-null trophoblast differentiation

Western blot of AKT phosphorylated at thr308, thr450, and ser473 in addition to total AKT and β -Actin during standard differentiation of wild-type and Sirt1-null trophoblasts.

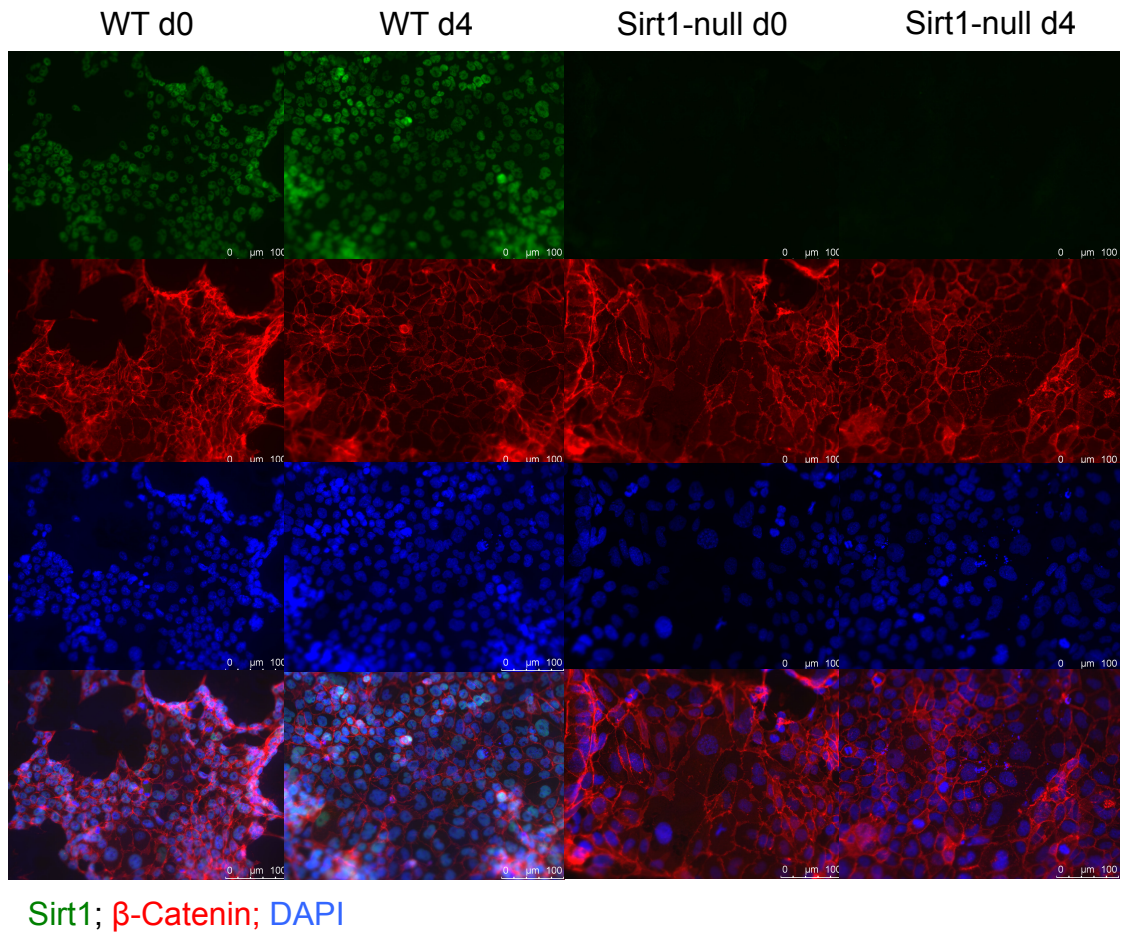


Figure 5.13 Localization of β -Catenin during differentiation is not affected by loss of Sirt1

Immunofluorescence of Sirt1 (green), β -Catenin (red) and DAPI of undifferentiated and differentiated (day 4) wild-type and Sirt1-null trophoblasts. No changes in the localization of β -Catenin were observed in either cell line.

Similar to β -catenin, Notch signaling is also important in mouse placental development and can be regulated by Sirt1. In mouse, Notch2 is important for TGC formation while deletion of Notch2 can compromise the maternal blood spaces in the placenta, leading to embryonic lethality (Hunkapiller et al., 2011; Gasperowicz et al., 2013). Notch signaling activates the transcription factor Hes1. qRT-PCR of Hes1 in differentiated WT and null trophoblasts demonstrated that Hes1 was higher in differentiated Sirt1-null trophoblasts, compared to WT cells, on days 5 to 7 (Figure 5.14).

5.5 Global gene expression profiling of WT and Sirt1-null trophoblasts

Gene expression profiling of WT and Sirt1-null trophoblasts was performed in an attempt to identify global gene pattern changes and to facilitate discovery of pathways regulated by Sirt1 in trophoblasts. Microarray-based analyses were performed on RNA from two different sets of WT and Sirt1-null TS cells on d0, 1, 2, 5, and 7. A principle component analysis (PCA) of the combined data sets confirmed the differentiation defect observed at the qRT-PCR level (Figure 5.15). The PCA is an unbiased method to separate samples based on the variation in expression pattern of the probes (genes) in the microarray. Without applying any statistics, PCA1, which accounts for 24% of the variability between the samples, separated both WT and null samples by days of differentiation. Furthermore, it is clear that the Sirt1-null d0 samples aligned more closely with WT d1-d2, while Sirt1-null d5-d7 samples did not differentiate as far as WT d5-d7. PCA2, which explained 15% of the variation in the samples, separated samples by presence or absence of Sirt1.

To confirm expression of standard differentiation markers in this data set, I identified genes that were statistically significantly changing during differentiation of

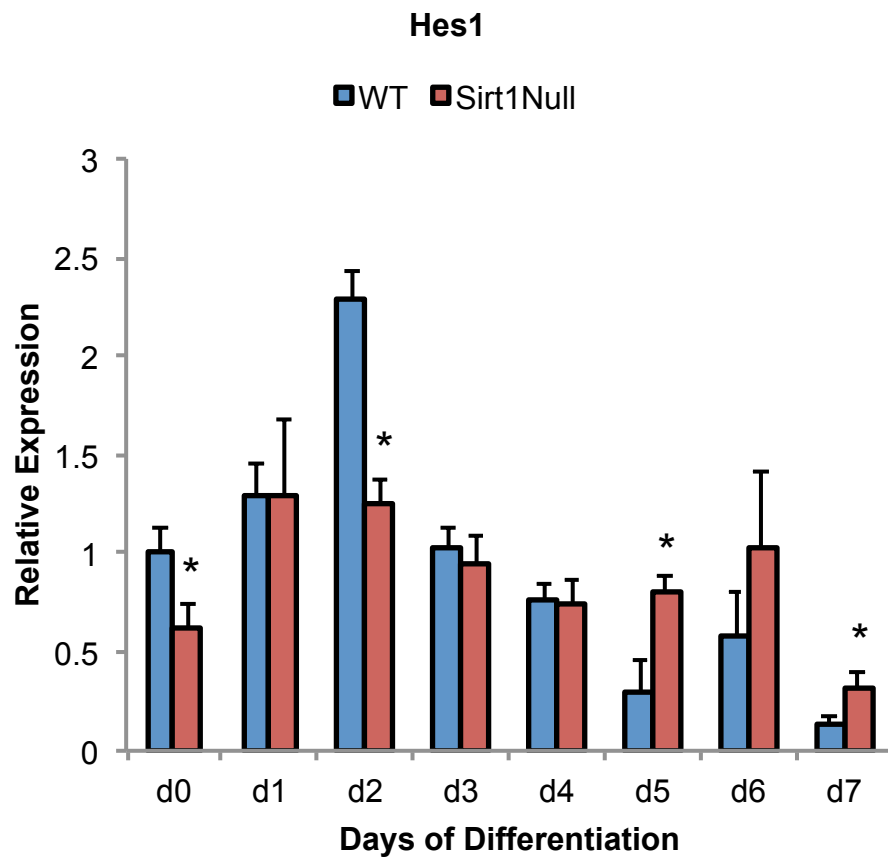


Figure 5.14 Expression of Hes1, a canonical Notch pathway target

qRT-PCR of Hes1 during wild-type and Sirt1-null trophoblasts. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

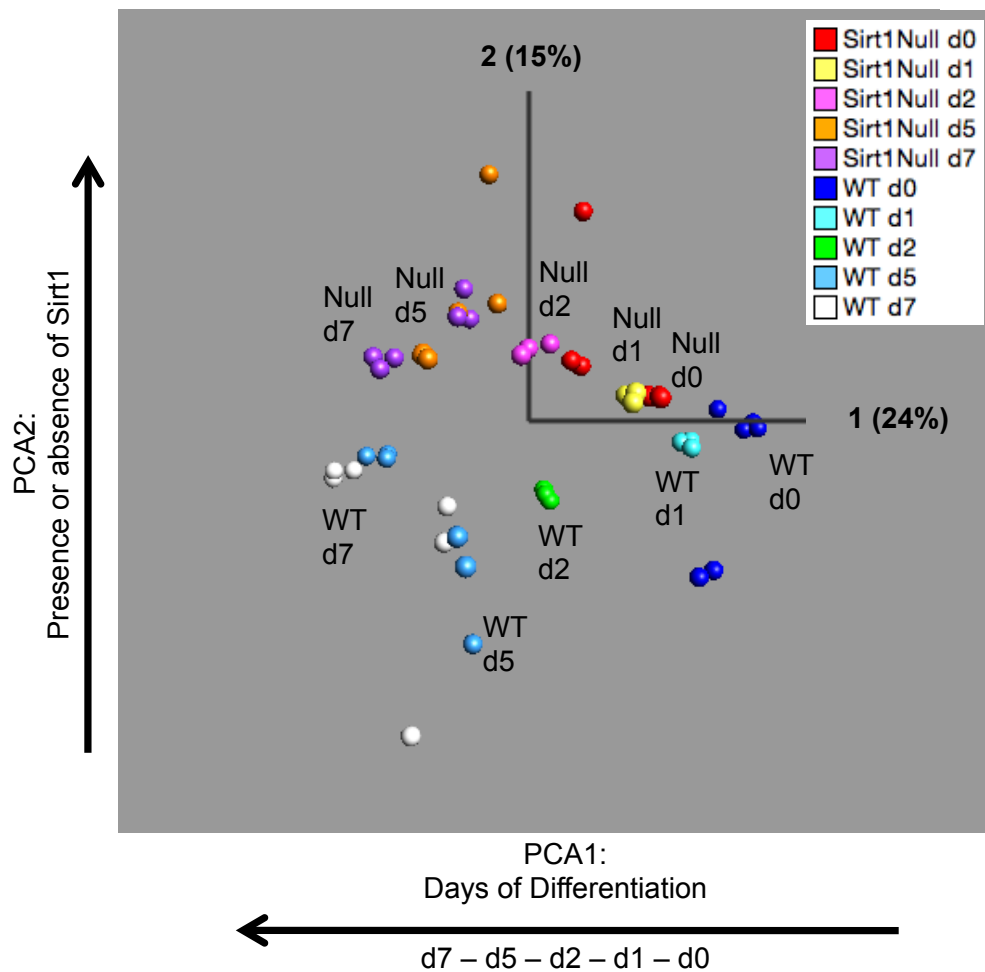


Figure 5.15 Principle component analysis of wild-type and Sirt1-null trophoblasts during differentiation

Principle component analysis (PCA) of microarray data from wild-type, wild-type #2 (43E2), Sirt1-null and Sirt1-null #2 (29B) trophoblasts at differentiation timepoints: day 0, 1, 2, 5, and 7. PCA 1 (24%) separates samples by differentiation and PCA 2 (15%) separates samples by genotype. No statistics were applied.

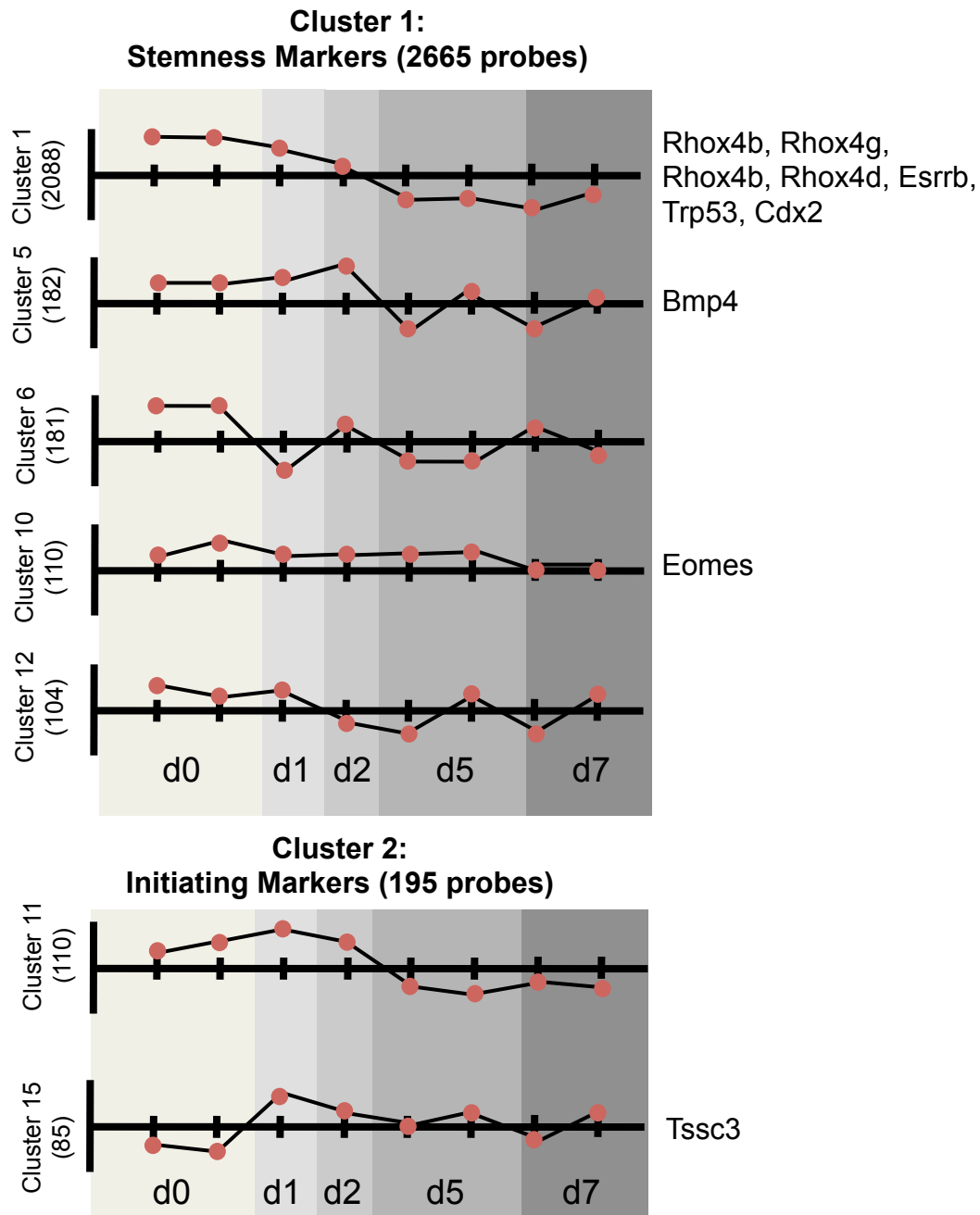


Figure 5.16 CLICK analysis of probes downregulated in WT trophoblast differentiation

Of probes statistically significantly changing during WT trophoblast differentiation, CLICK analysis identified clusters of genes that were either downregulated with differentiation (Cluster 1) or upregulated at early timepoints (Cluster 2). Biologically relevant genes were found in these clusters.

WT cells. These genes were then subjected to a CLICK analysis, which is an unbiased analysis that clusters probes (genes) together based on similar expression patterns. 2665 probes were identified to be downregulated with differentiation while 195 probes peaked at early timepoints; these groups of genes should represent either stemness or differentiation initiation markers, respectively (Figure 5.16). Identification of standard stemness markers such as *Cdx2*, *Esrrb* and progenitor markers such *Rhox4* and *Phlda2/Tssc3* were found in these groups, confirming the validity of the combined data-sets. Similarly, 2823 probes were determined to be upregulated during WT differentiation and within this set of probes were genes such as *Ctsq*, *Prl3b1* (PL-I) and *PPARg*, amongst others (Figure 5.17).

5.6 Identification of signaling pathways dysregulated in Sirt1-null trophoblast stem cells

After validating the microarray data, I used this data set of global gene expression to identify signaling pathways and cellular functions dysregulated in trophoblasts in the absence of Sirt1. In order to do this, I evaluated gene expression differences between WT and Sirt1-null TS cells on d0 in cells in the undifferentiated state. First, I identified genes that were statistically significantly differentially expressed between WT and Null d0 cell samples.

A pathway analysis of probes downregulated in Sirt1-null TS cells (886 gene probes), compared to WT TS cells, identified several interesting pathways. Of note, canonical signaling pathways downregulated included protein ubiquitination pathway, cell cycle regulation, inflammation and BMP signaling (Figure 5.18). Gene ontology analyses identified similar categories of genes enriched in genes downregulated in

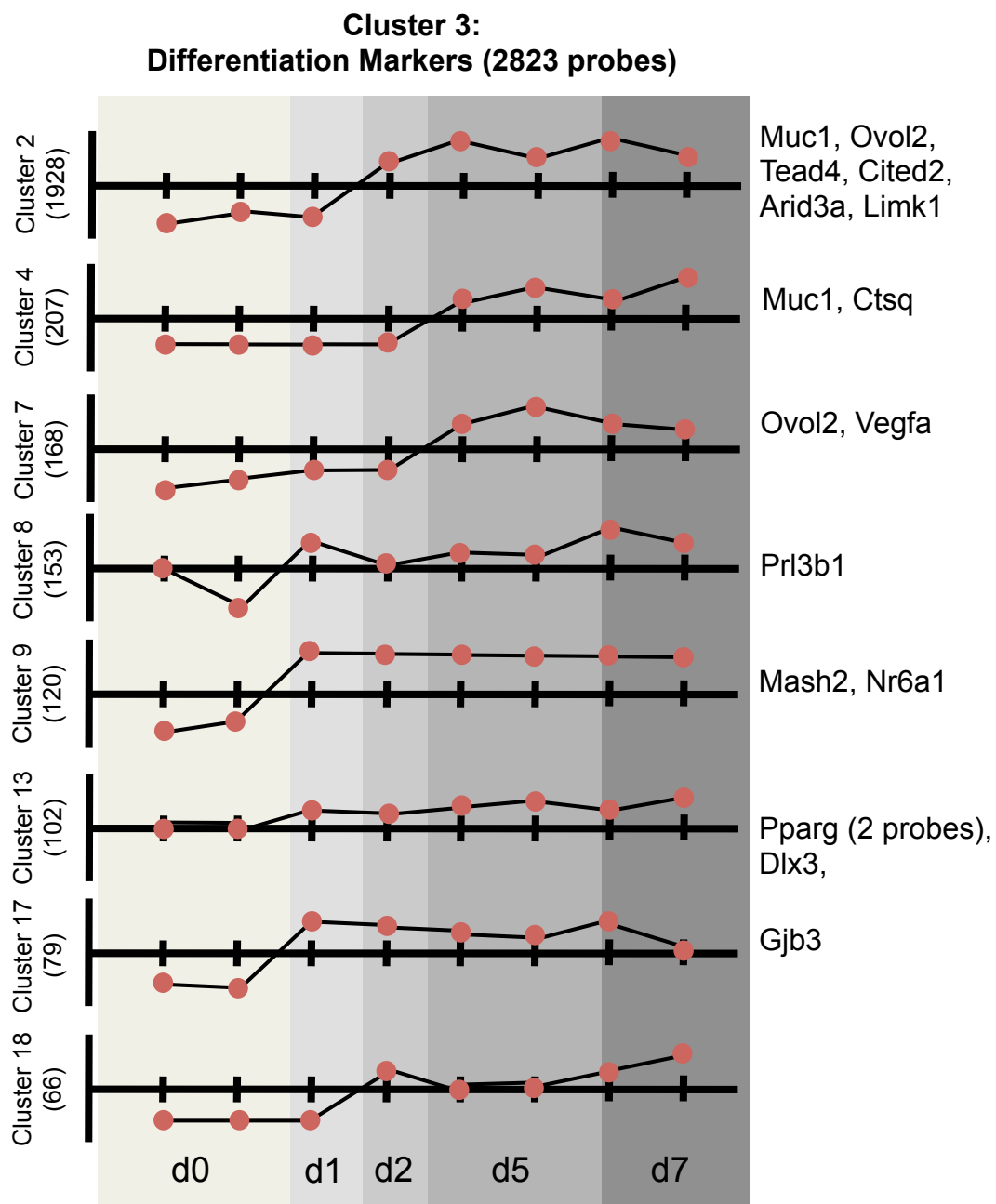


Figure 5.17 CLICK analysis of probes increasing in WT trophoblast differentiation

Of probes statistically significantly changing during differentiation, CLICK analysis identified clusters of genes that were upregulated with differentiation (Cluster 3) and included genes known to be important in trophoblast differentiation.

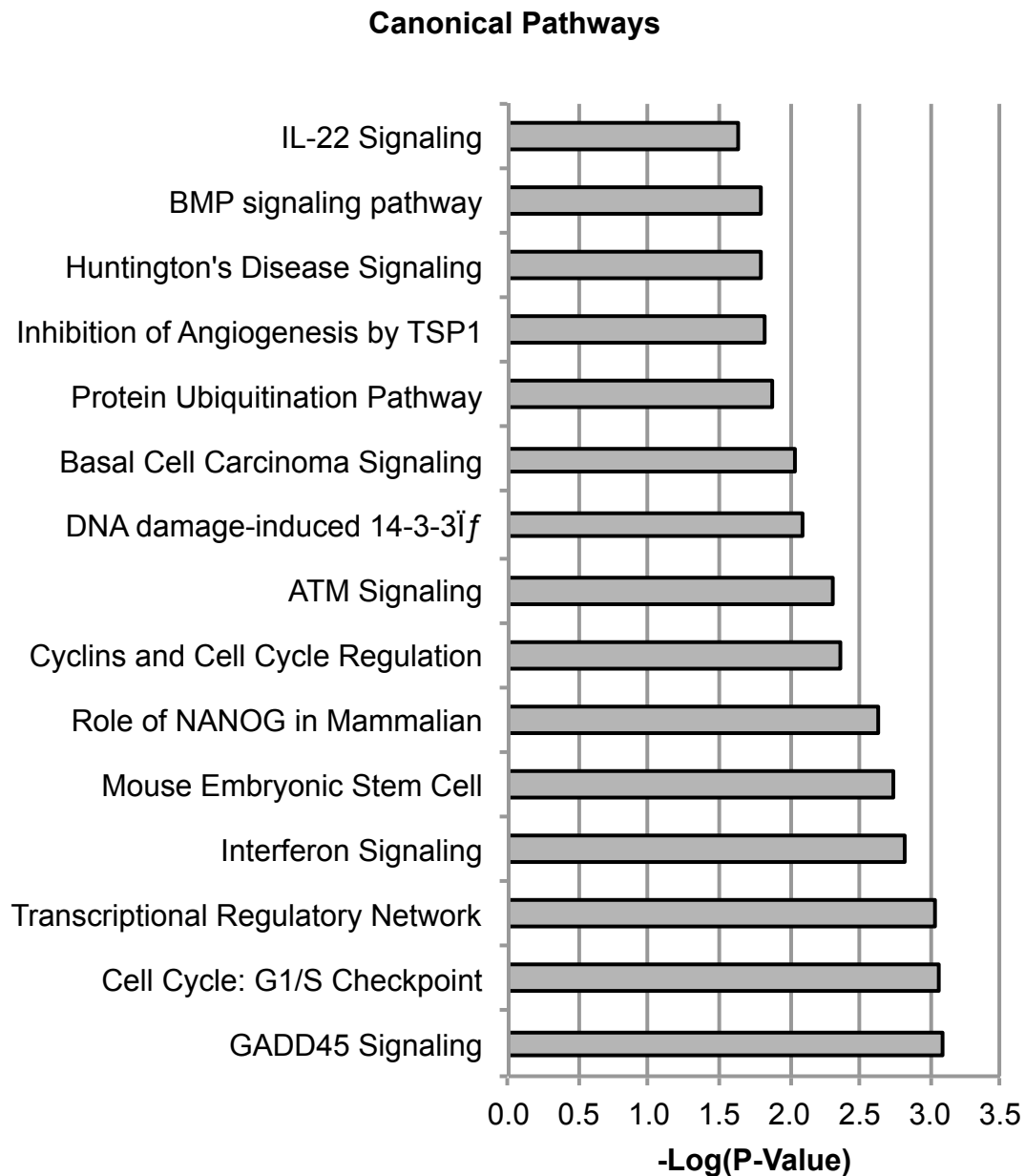


Figure 5.18 Canonical pathways enriched in genes downregulated Sirt1-null d0
 Canonical pathway analysis of the 886 genes downregulated in null d0 TS cells. Some of these pathways can be associated with known functions of Sirt1 and cell stemness.

null d0 cells, such as cell cycle and inflammation, in addition to vasculature development (Figure 5.19).

Concurrently, I did a similar analysis of probes (genes) upregulated in undifferentiated null trophoblasts on d0 of differentiation (873 probes). Amongst the canonical pathways upregulated included HGF signaling along with pathways in response to oxidative stress and inflammatory pathways (Figure 5.20). Classical gene ontology supported the canonical pathway analysis, highlighting alterations in cellular response pathways such as the p53 pathway, hypoxia signaling and cell growth (Figure 5.21).

Chapter 5, in part, contains material that is currently being prepared for submission for publication of the material. Arul Nambi Rajan Kanaga, Soncin Francesca, Moretto-Zita Matteo, Iyer Pooja, Tache Veronique, Pizzo Donald, McBurney Michael, Parast Mana M. The dissertation author was the primary investigator and author of this material.

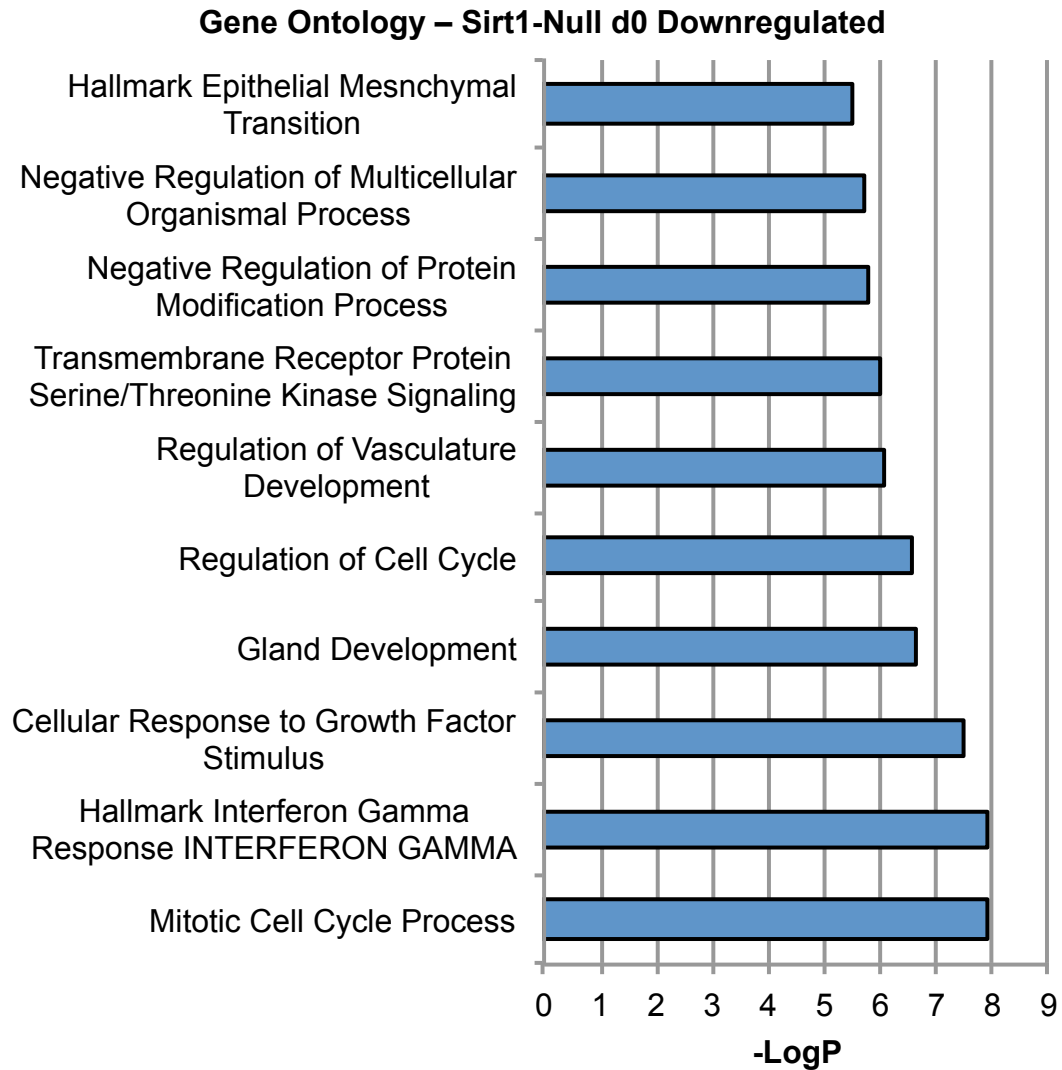


Figure 5.19 Gene ontology of genes downregulated in Sirt1-null d0 cells

Gene ontology of gene probes downregulated in null d0 TS cells. Some of these ontologies can be associated with known functions of Sirt1 and the observed Sirt1-null phenotype.

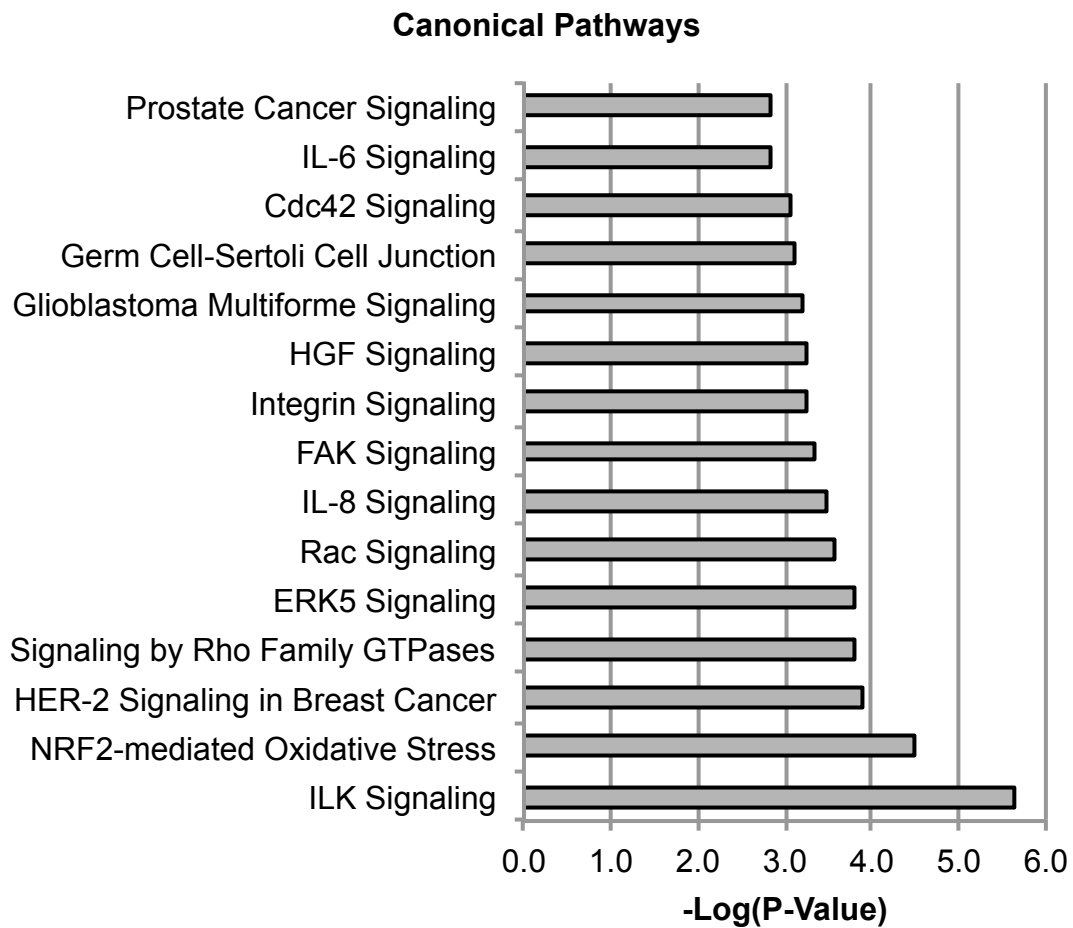


Figure 5.20 Canonical pathways enriched in genes upregulated Sirt1-null d0
 Canonical pathway analysis of the 873 genes probes upregulated in null d0 TS cells. Some of these pathways can be associated with known functions of Sirt1 in other models and includes HGF Signaling.

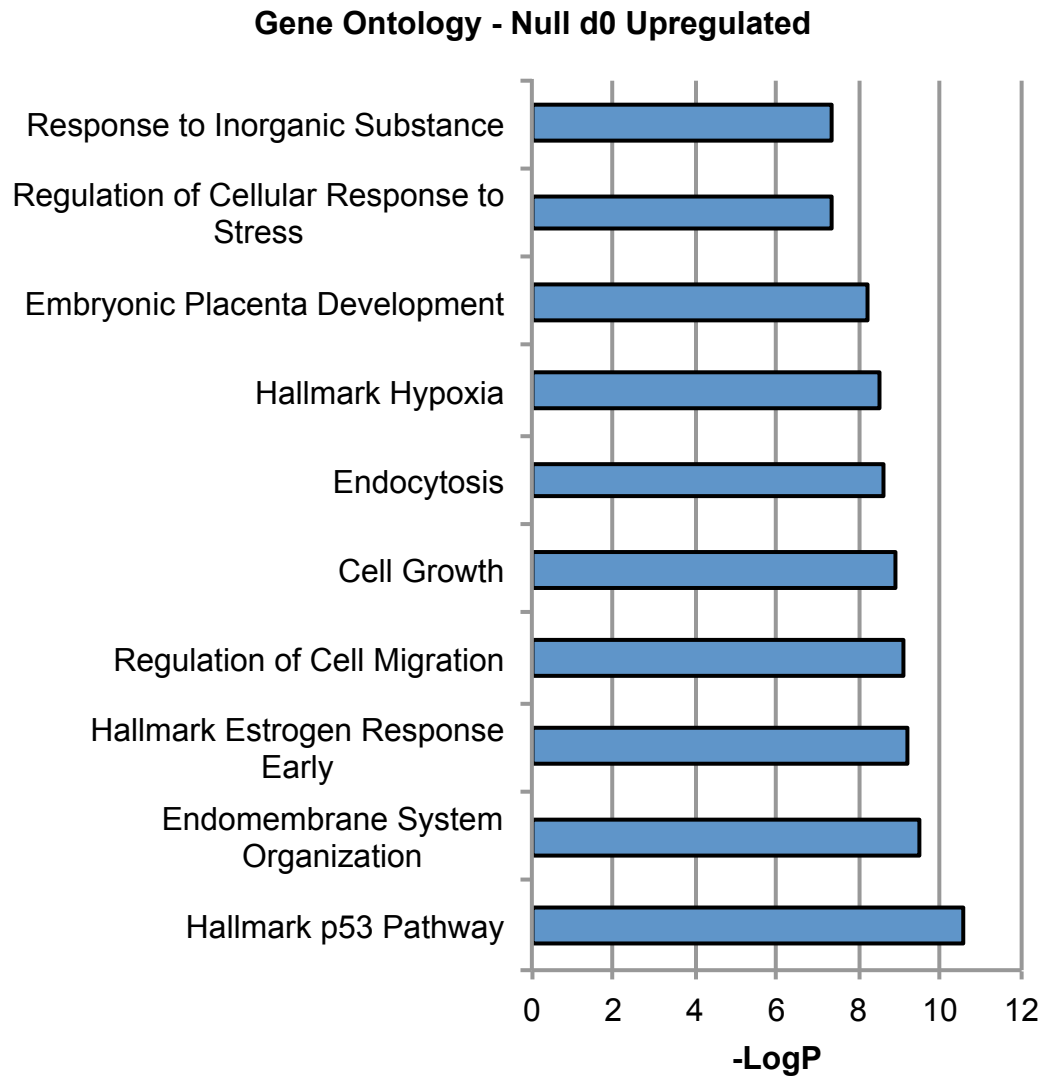


Figure 5.21 Gene ontology of genes upregulated in Sirt1-null d0 cells

Gene ontology of gene probes upregulated in null d0 TS cells. Some of these categories can be associated with known functions of Sirt1, such as the p53 pathway as well as biological relevance to the current model, such as embryonic placenta development.

Chapter 6

The Role of Sirt1 Activity in Mouse Trophoblast Differentiation and Placental Development

6.1 Introduction

Several Sirt1 activators and inhibitors have been identified and used to determine the role of Sirt1 activity in various cellular processes. Resveratrol is widely used to activate Sirt1 *in vitro* in cells such as cardiomyocytes (Chen et al., 2009) and mesenchymal stem cells (Shakibaei et al., 2012). Sirtinol (Balaiya et al., 2012; Kim et al., 2012) and Ex527 (Di Emidio et al., 2014; Pan et al., 2016) are two of several Sirt1 inhibitors often used in such studies. Furthermore, Sirt1-mutant mice have also been derived on the 129/Sv background and used to demonstrate that Sirt1's deacetylase catalytic domain is necessary in several tissues (Seifert et al., 2012). Though these mice have a full-length Sirt1 protein, there is a single point mutation in the known Sirt1 catalytic domain. These mice were reported to survive but exhibit fetal growth restriction similar to Sirt1-null pups (McBurney et al., 2003). Since all Sirt1 deacetylation activity reported is through its standard catalytic domain, we hypothesized that the differentiation defect observed in trophoblast lacking Sirt1 was due to lack of Sirt1 activity. To confirm that Sirt1 activity is responsible for our observed phenotype, I used Sirt1 activators and inhibitors as well as the Sirt1-mutant mice to evaluate whether Sirt1's role in trophoblast is dependent on an intact catalytic domain.

6.2 Modulation of Sirt1 activity using Sirt1 small molecules

Absence of Sirt1 causes a differentiation defect in trophoblast. In other systems, Sirt1's effects have been attributed to its known catalytic function. I therefore hypothesized that treatment of WT-TS cells with Sirt1 inhibitors (Sirtinol and Ex527) would recapitulate the *in vitro* Sirt1-null differentiation defect. Both wild-type cell lines (WT and 43E2) were treated with either 50 μ M Sirtinol or 100 μ M Ex527. I

then evaluated if these inhibitors affected differentiation of WT TS cells. In both WT lines, the presence of Sirtinol and Ex527 did not inhibit differentiation, as indicated by a decrease in *Esrrb* (Figure 6.1A). However, inhibitors did not produce reproducible changes in expression of differentiation markers. Though Ex527 reduced *Tpbpa* in both WT cell lines, Sirtinol appeared to increase expression of *Tpbpa* in one WT cell line while inhibiting it in a second WT cell line (43E2) (Figure 6.1B). Results were similarly inconsistent for labyrinthine markers. Ex527 treatment did not significantly affect *SynA* expression in either cell line, while Sirtinol increased *SynA* expression in one line and reduced it in the second line (43E2) (Figure 6.1C).

Resveratrol is commonly used to directly activate Sirt1. To identify an effect of Sirt1 activation in trophoblast, WT TS cells were treated with 25 μ M resveratrol for five days in either growth or differentiation conditions. Null TS cells were also treated to identify Sirt1-dependent effects. In growth media, both WT and null cells treated with resveratrol exhibited only a slight decrease in *Esrrb* (Figure 6.2A). However, there was a significant increase in *Tpbpa*, but not *SynA*, expression with Sirt1 activation (Figure 6.2B). Since absence of Sirt1 prevents proper *in vitro* differentiation of trophoblasts under differentiation conditions, I also treated WT and Null TS cells with resveratrol during differentiation. Resveratrol treatment did not interfere with differentiation, based on reduction in *Esrrb* expression (Figure 6.3A). Interestingly, though WT TS cells and cells treated with carrier (DMSO) alone exhibited an increase in *Tpbpa*, WT cells treated with 25 μ M resveratrol had significantly reduced levels of this marker (Figure 6.3B). Furthermore, these same cells had a more dramatic increase in *SynA* expression compared to DMSO control cells (Figure 6.3C). This

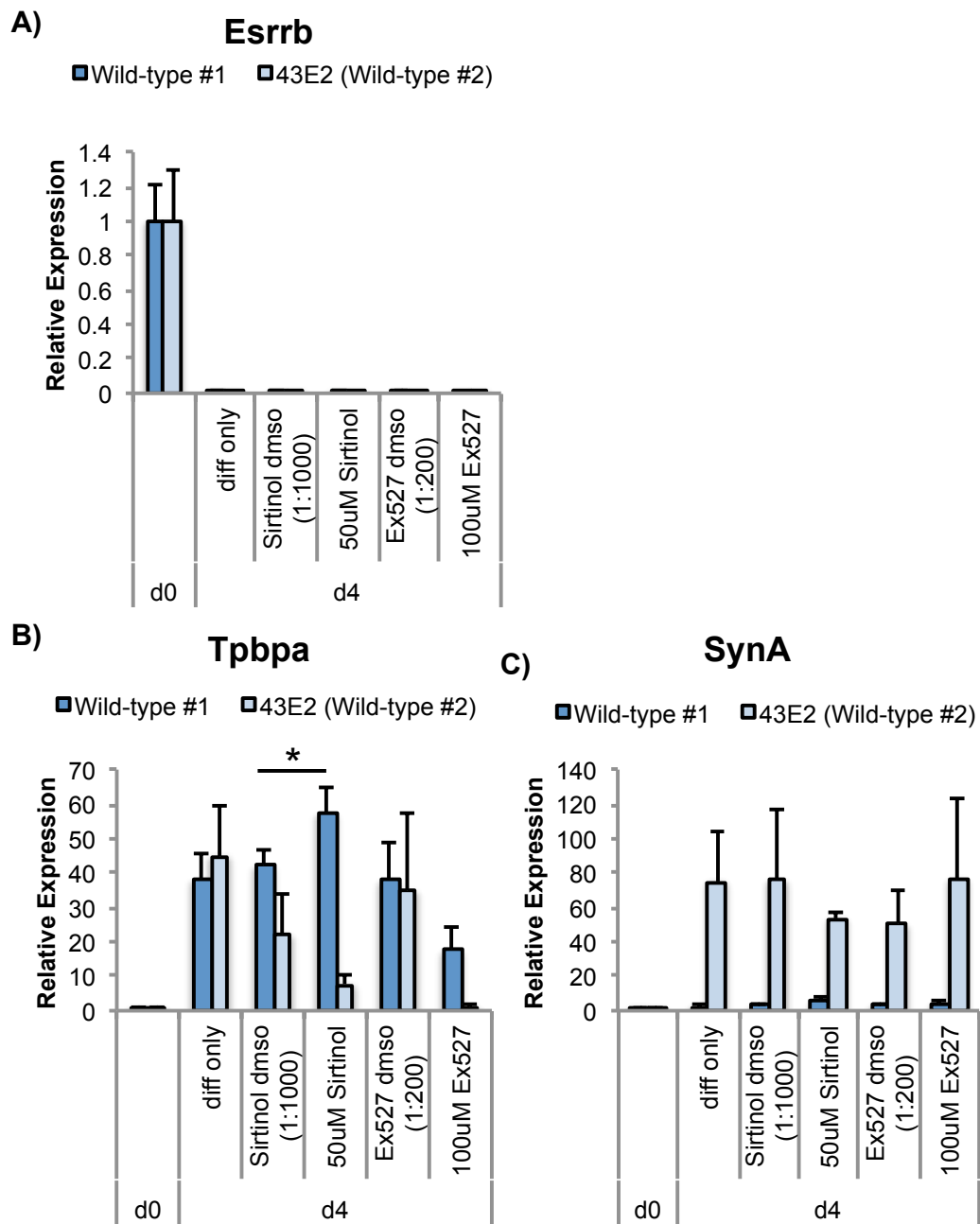


Figure 6.1 Wild-type TS differentiation in the presence of Sirt1 small molecule inhibitors

Wild-type #1 and wild-type #2 (43E2) differentiated in the presence of either 50 μ M Sirtinol, 100 μ M Ex527 or dms vehicle. qRT-PCR for TS markers (A), *Tpbpa* for spongiotrophoblasts (B) and *SynA* for labyrinthine (C). Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

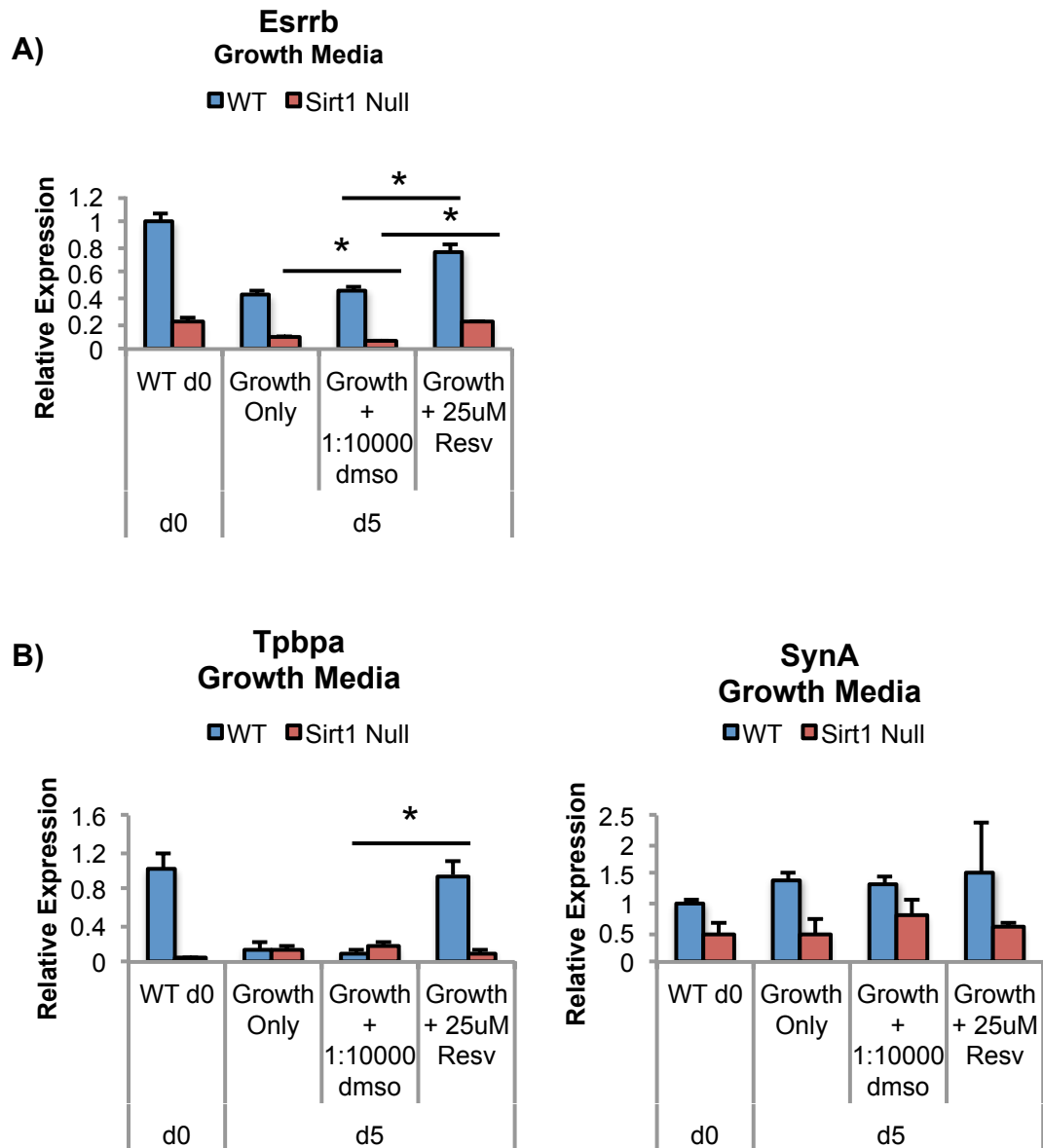


Figure 6.2 Effect of Resveratrol on TS cells in growth media

Wild-type and Sirt1-null TS cells were maintained in growth media in the presence of 25 μ M resveratrol. qRT-PCR for Esrrb (A) as well as spongiotrophoblast marker Tpbpa and labyrinthine marker SynA (B) indicates differentiation effect of resveratrol. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

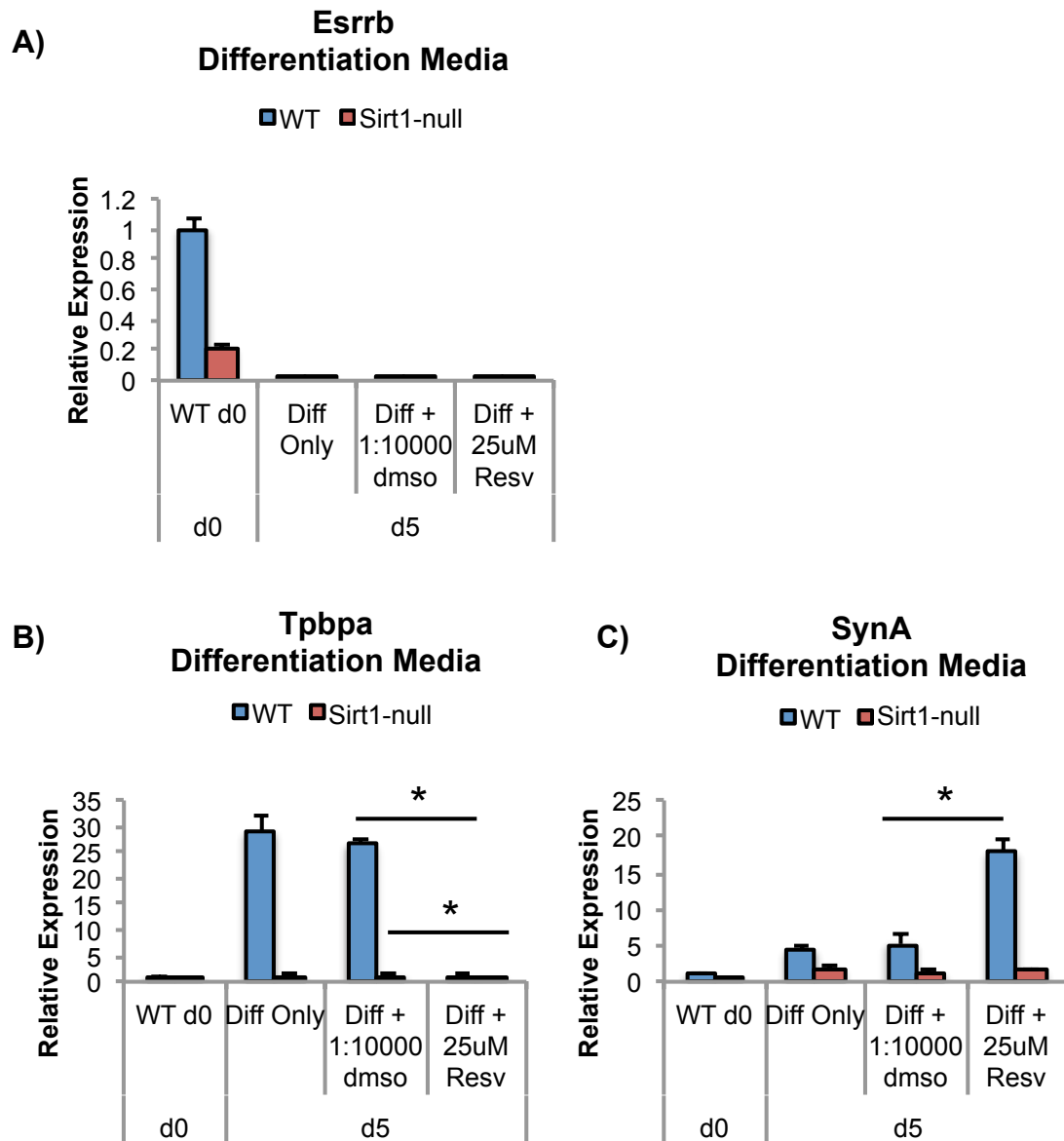


Figure 6.3 Resveratrol promotes differentiation into labyrinthine trophoblasts

Wild-type and Sirt1-null TS cells were maintained in differentiation media in the presence of 25 μ M resveratrol. qRT-PCR evaluated differentiation for TS marker (A) and differentiation markers of spongiotrophoblasts and labyrinthine trophoblasts (B). Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

enrichment in of labyrinthine marker expression only occurred in the WT TS cells and was not observed in null TS cells.

6.3 Derivation and characterization of Sirt1-Mutant Trophoblasts

In addition to modulation of Sirt1 activity using small molecules, I also derived two Sirt1-mutant trophoblast stem cell lines from e3.5 Sirt1-mutant blastocysts. The first of these lines is Sirt1-mutant line #1 (55C) which was derived with a new WT line (55D) from a littermate blastocyst. To assess if a catalytically inactive Sirt1 affects TS differentiation, these new TS lines were differentiated for seven days and standard differentiation markers were evaluated by qRT-PCR. Compared to previously derived WT cells, both the new WT (55D) and Sirt1-mutant (55C) cells were both more differentiated on d0 at the undifferentiated TS state based on the reduced *Esrrb*; however *Esrrb* decreased with differentiation in all of the lines (Figure 6.4A). *SynA* expression was increased significantly in the mutant line compared to both new and old WT cells (Figure 6.4B). Though *Tpbpa* expression increased on d7 of all three cell lines, it was highest in the new WT (55D) cells (Figure 6.5A). Similar expression patterns were observed for TGC markers *Prl3b1* and *Prl2c2*, though both of these markers, and *Tpbpa*, were all slightly higher on d0 of the new WT (55D) cells (Figure 6.5B).

Sirt1-mutant line #2 (A42B) was derived and compared to our secondary WT (43E2) TS cells to confirm the Sirt1-mutant cell phenotype. Even in the undifferentiated TS state, mutant line #2 had significantly reduced levels of *Esrrb* expression though, this marker did decrease further with differentiation (Figure 6.6A). Though WT cells showed an increase in labyrinthine markers, neither *Gcm1* nor *SynA* increased in the mutant cells, though *SynA* was higher on mutant line #2 d0

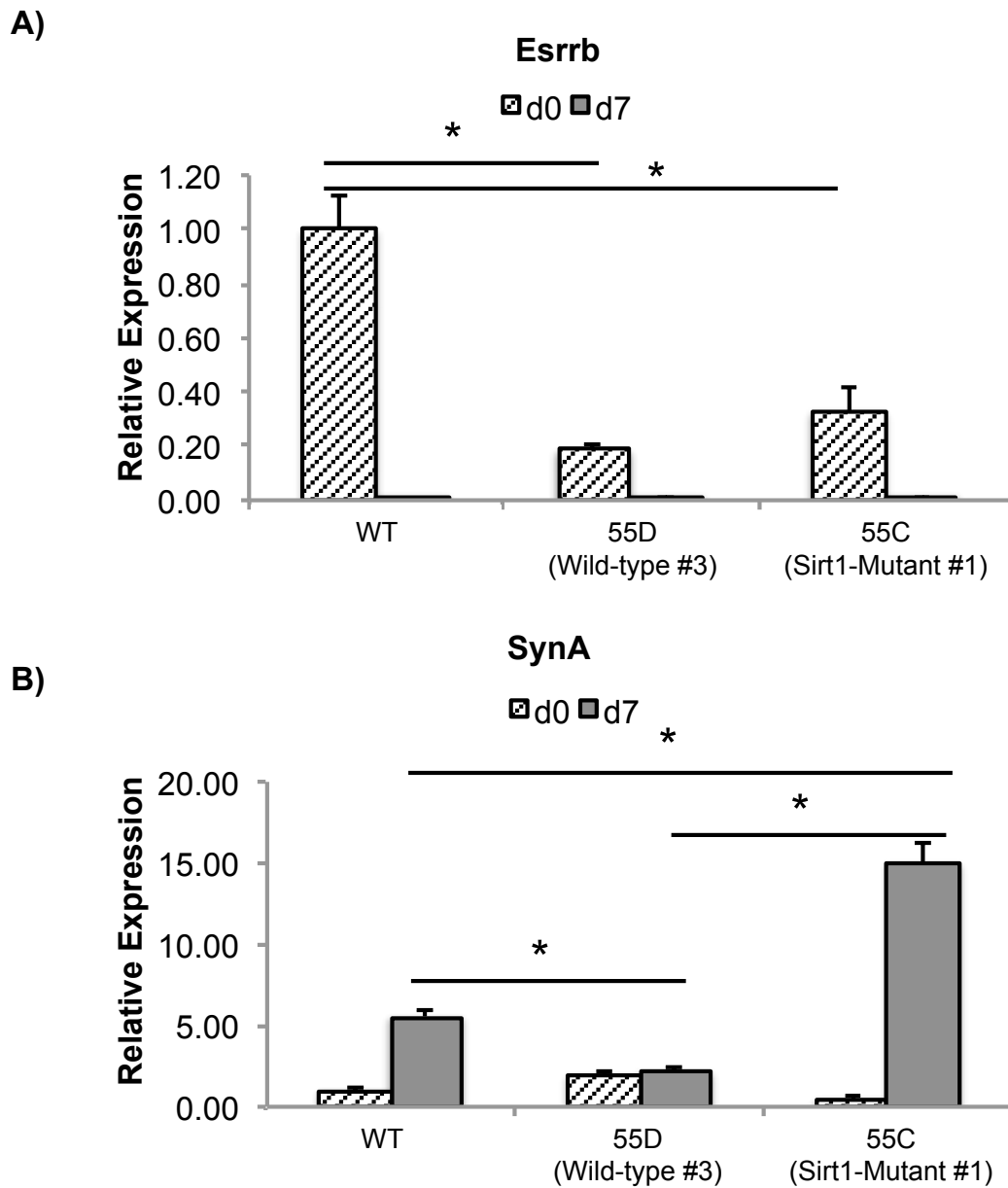


Figure 6.4 Comparison of Wild-type #3 and Sirt1-Mutant #1 TS cell lines

55D (wild-type #3) and 55C (Sirt1-Mutant #1) littermates TS cell lines were derived and compared to wild-type #1 TS cells. qRT-PCR was used to evaluate differentiation. (A) *Esrrb* demonstrated ability of these cells to differentiate compared to original WT TS cells. (B) *SynA* expression shows these TS cell lines ability to differentiate into labyrinthine lineage. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

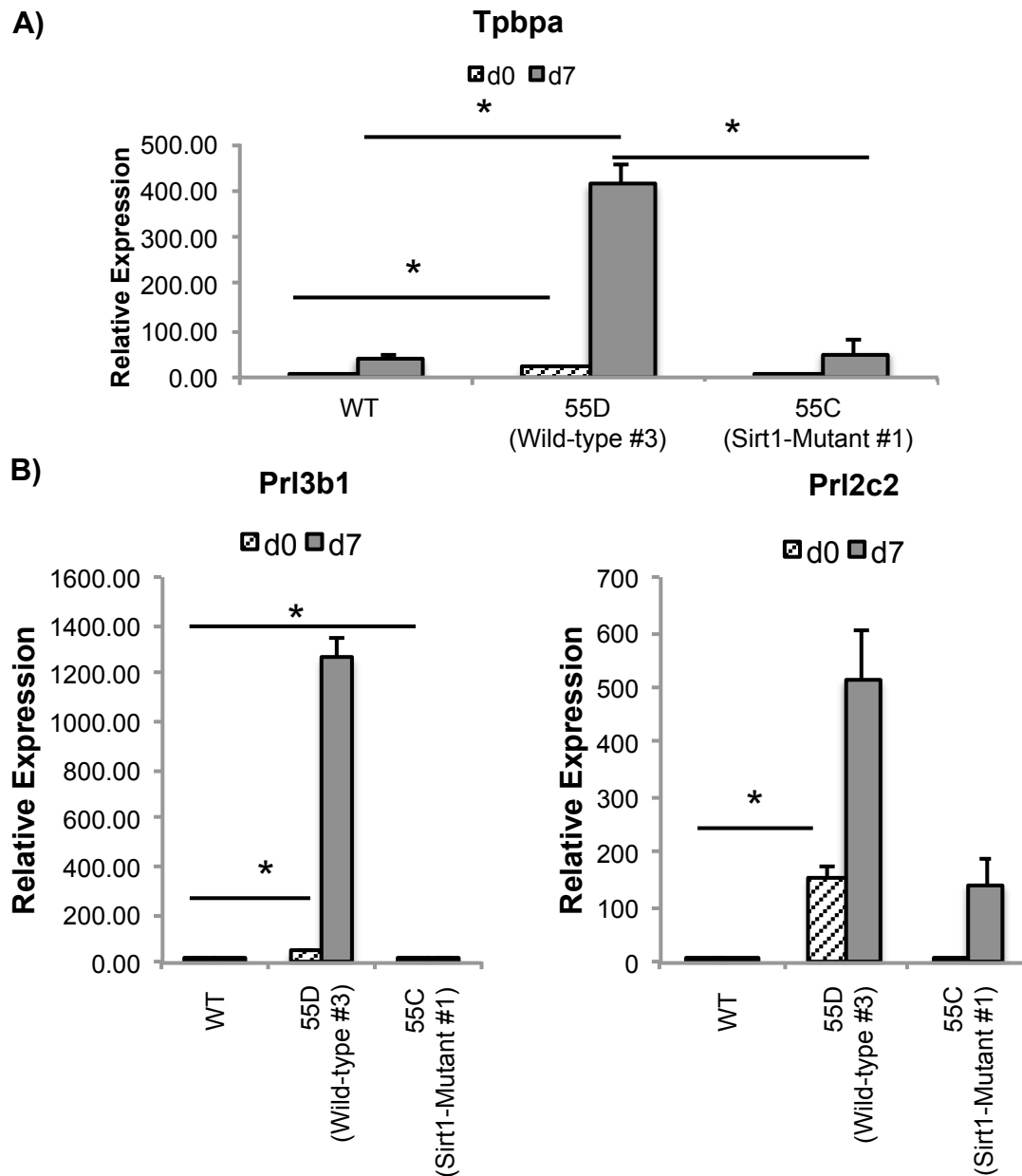


Figure 6.5 Differentiation of 55D (wild-type #3) and 55C (Sirt1-Mutant #1) TS cell lines into invasive trophoblasts

55D (wild-type #3) and 55C (Sirt1-Mutant #1) littermates TS cell lines were derived and compared to wild-type #1 TS cells. qRT-PCR was used to evaluate differentiation. (A) *Tpbpa* demonstrates differentiation into spongiotrophoblasts. (B) *Prl3b1* and *Prl2c2* show differentiation into TGC. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

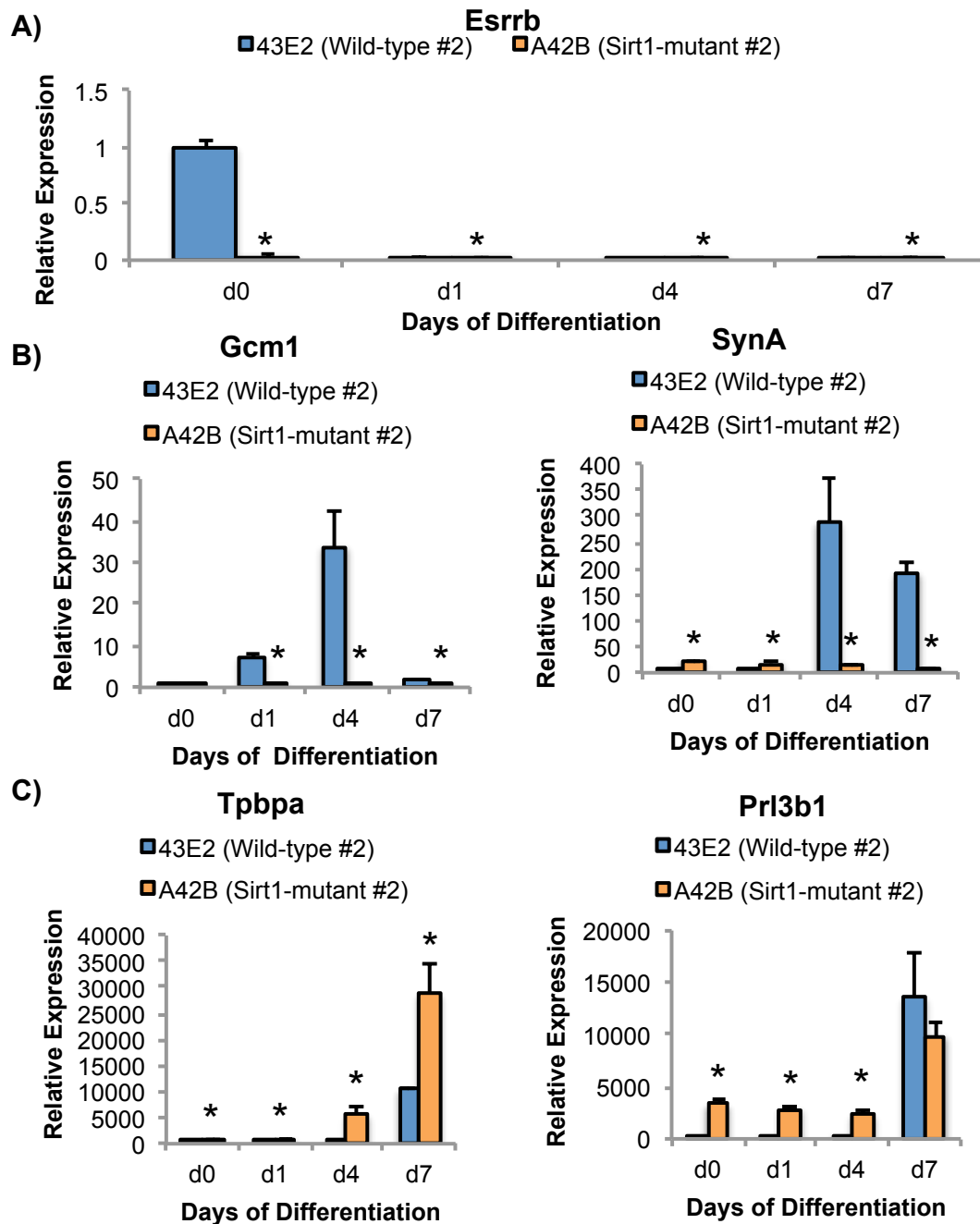


Figure 6.6 Differentiation of 43E2 (Wild-type #2) and A42B (Sirt1-Mutant #2) TS cell lines

A42A (Sirt1-Mutant #A) TS cell lines were compared to 43E2 (wild-type #2) TS cells. qRT-PCR evaluated differentiation. (A) *Esrrb* shows the stemness of line during differentiation. (B) Expression of early and late labyrinthine markers and (C) spongiotrophoblast and TGC markers. Values adjusted to WT d0 and normalized to 18S ribosomal RNA. * $p < 0.05$.

(Figure 6.6B). Invasive trophoblast markers *Tpbpa* and *Prl3b1* were both higher on mutant #2 d0 and increased with differentiation on d7, with *Tpbpa* expressed at a significantly higher level compared to WT cells (Figure 6.6C).

6.4 Characterization of Sirt1-Mutant embryos and placentas

Since Sirt1-null placentas and embryos are growth restricted, I analyzed Sirt1-mutant placentas and embryos to determine if Sirt1 catalytic function contributes to changes in the mouse phenotype. Sirt1-null embryos exhibit embryonic lethality at e13.5. I therefore first examined homozygous mutant embryos at this same developmental timepoint. No differences were observed in the embryonic weight of mutant embryos (Figure 6.7A). Further examination of placental weight and embryo-to-placenta ratios also demonstrated no significant difference between homozygous mutant mice and their WT littermates (Figure 6.7 B and C). In addition, the number of mutant pups observed at this time point was also not statistically significantly reduced. Analysis of embryos and placentas at a later timepoint, e16.5, showed similar results in that no embryonic or placental growth restriction was observed (Figure 6.8).

Though no growth restriction was observed, I evaluated the mutant placentas to determine if placentas are histologically normal in the absence of a catalytically active Sirt1. Sirt1-null placentas exhibit greatest developmental defects in the labyrinthine lineage. Inspection of the mutant placentas and the labyrinth demonstrated no significant differences between WT and mutant placentas (Figure 6.9). Immunohistochemistry of CD31, to identify the labyrinthine layer, and in situ hybridization for the spongiotrophoblast marker *Tpbpa* did not identify any observable defects in the mutant placentas (Figure 6.10A). Pan-Cytokeratin staining was done to

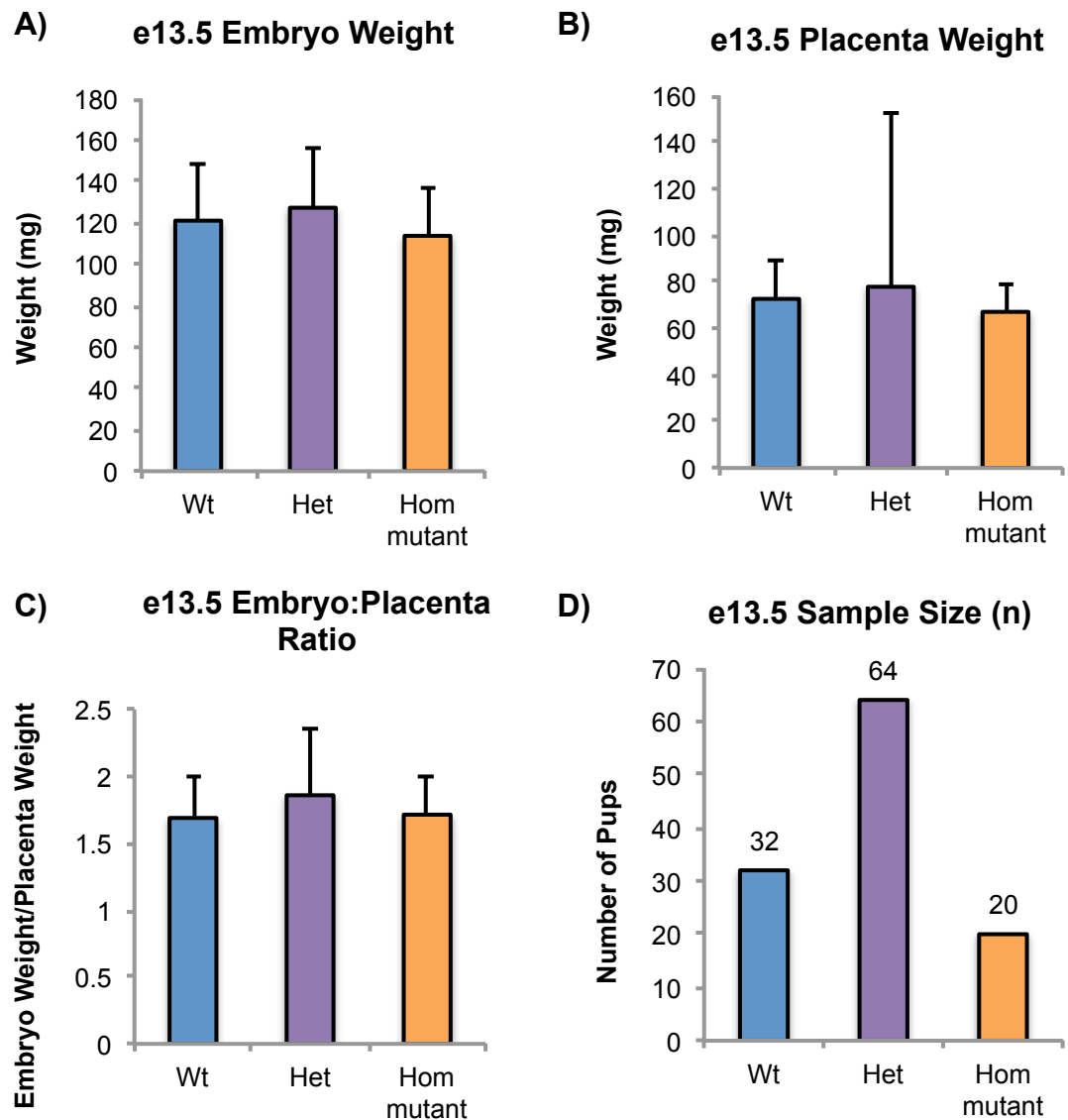


Figure 6.7 Sirt1-Mutant embryos and placentas at e13.5 are not different from WT

Heterozygous Sirt1-mutant mice were mated and resulting embryos and placentas were evaluated at e13.5 (A) Embryo and (B) placental weights were measured at time of dissection. (C) Embryo-placenta ratio was calculated. (D) Number of pups of each genotype were counted. * $p < 0.05$.

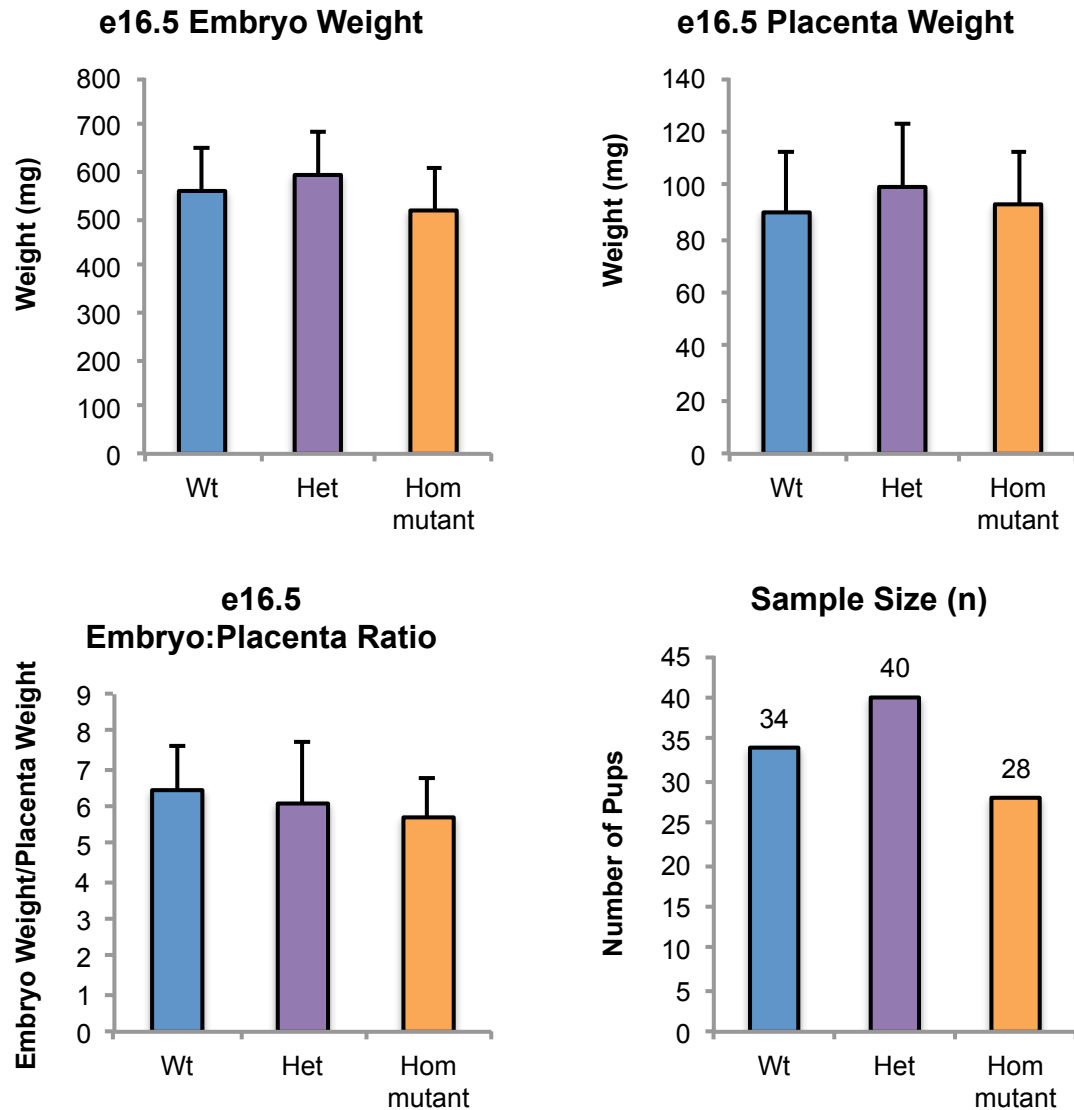


Figure 6.8 Sirt1-Mutant embryos and placentas at e16.5 are not different compared to WT

Heterozygous Sirt1-mutant mice were mated and resulting embryos and placentas were evaluated at e16.5. Embryo and placental weights were measured at time of dissection, embryo-placenta ratio was calculated and number of pups of each genotype were counted. * $p < 0.05$.

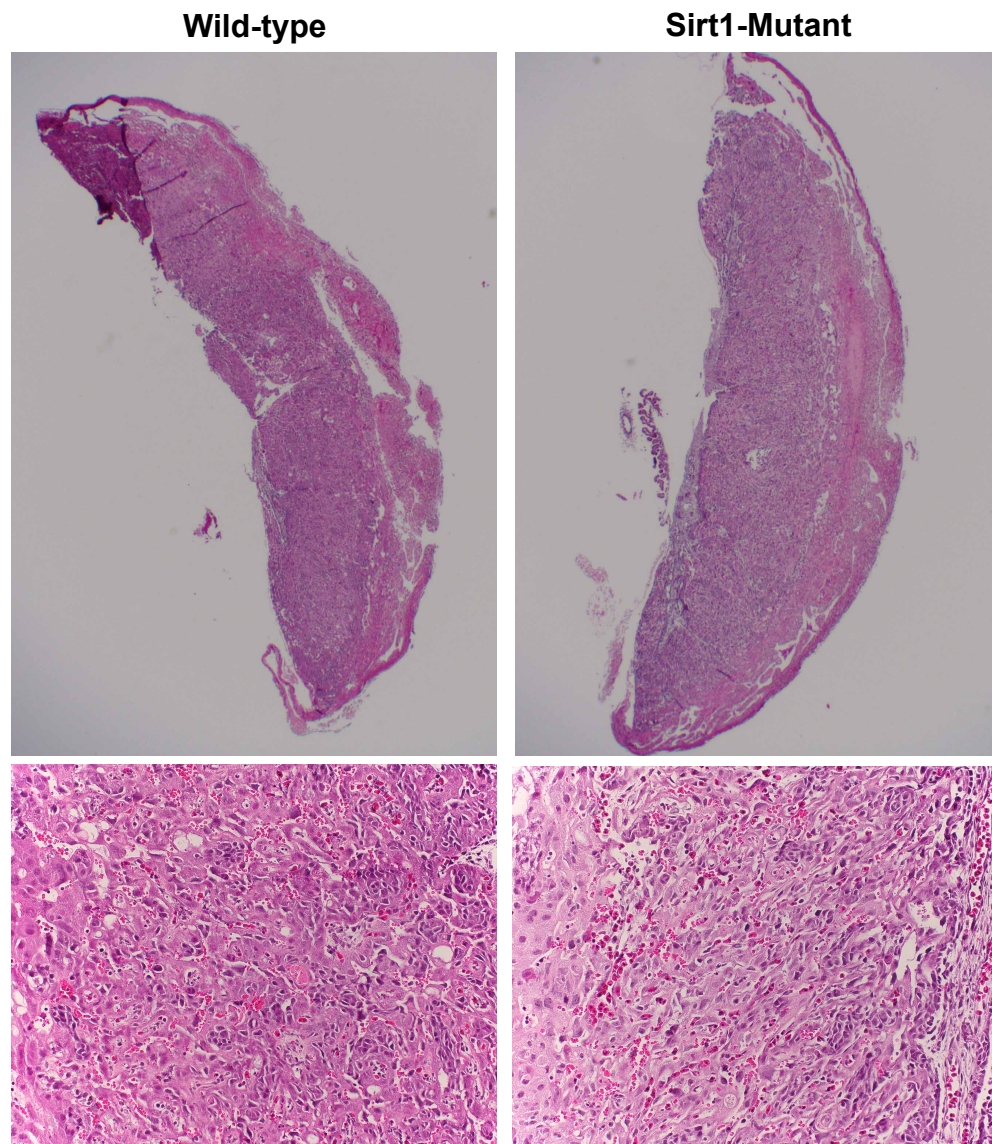


Figure 6.9 Sirt1-Mutant placentas at e13.5 do not exhibit changes in morphology

H&E of e13.5 wild-type and homozygous Sirt1-mutant placentas were compared. No differences were observed in total placenta or in labyrinthine layer (bottom). Magnification is 20x (top) and 200x (bottom)

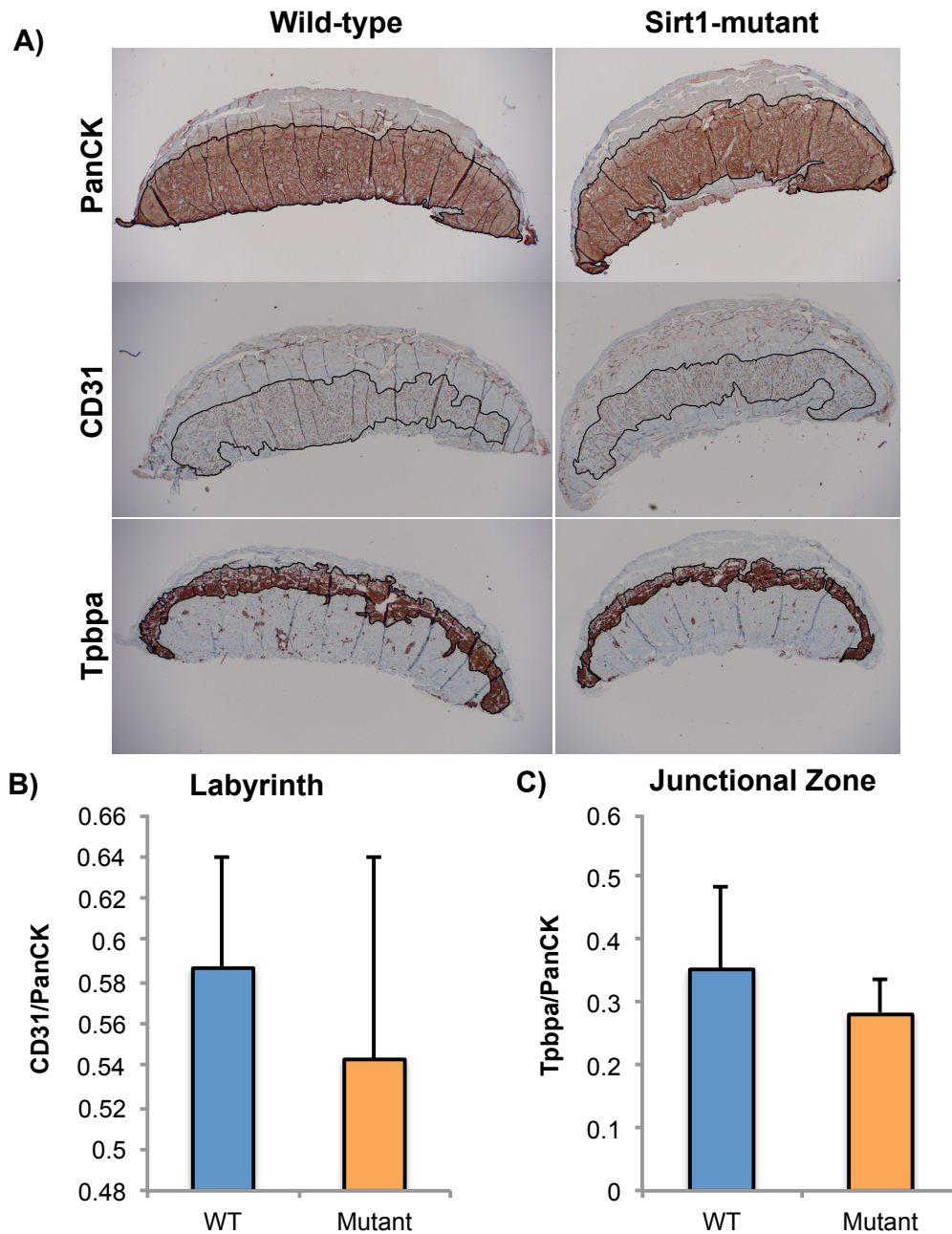


Figure 6.10 No observed differences in the spongiotrophoblast and labyrinth of Sirt1-Mutant placentas at e13.5

(A) IHC for pan-cytokeratin stained for all trophoblast. IHC for CD31 stained for vasculature and indicates the labyrinth. ISH for Tpbpa identified the junctional zone. (B) Area of labyrinth and junctional zone were measured and normalized to total placental area. Magnification is 20x. * $p < 0.05$.

determine the area of the entire area of the placenta in a section. When compared to the total area of the placenta, no difference was measured in the area of the labyrinth (Figure 6.10B) or spongiotrophoblast (Figure 6.10C).

Chapter 7

Discussion

7.1 In the absence of Sirt1, trophoblast stem cells show an altered pattern of differentiation

Sirt1 is studied in an array of tissues; however, the role of placental Sirt1 has not been studied. To elucidate the role of Sirt1 specifically in trophoblast, I isolated wild-type and Sirt1-null trophoblast stem cells from e3.5 blastocysts and maintained them *in vitro*. One of the most dramatic differences between the WT and Sirt1-null trophoblasts was the altered state of differentiation.

When cultured under “undifferentiated” conditions, Sirt1-null cells expressed reduced TS markers such as *Cdx2* and *Esrrb* compared to WT. Global gene expression profiling of these same cells confirmed that in the absence of Sirt1, TS cells were in fact more differentiated, with the null d0 cells aligning more closely with differentiated WT d1-d2 samples on principle component analysis. Gene ontology analysis of 873 probes upregulated in null d0 cells also showed enrichment of genes important for embryonic placental development, further supporting that null d0 cells are more differentiated. Gene ontology also highlighted an increase in genes involved in cell growth and regulation of cell migration, both characteristics of more differentiated trophoblast.

Part of this premature differentiation may be due to dysregulation of TGF β signaling and specifically BMP signaling. TGF β signaling is known to be important in placental development and trophoblast differentiation. TGF β and Activin have been shown to, in the presence FGF4, maintain undifferentiated TS cells (Erlebacher et al., 2004). In the absence of FGF4, Activin inhibits spongiotrophoblast/TGC and promotes differentiation into the labyrinthine lineage (Natale et al., 2009; Soncin et al., 2015). When I assessed Activin A signaling in WT and null TS cells, I observed

that null TS cells have a reduced response to Activin A, which likely contributes to their more differentiated phenotype.

The roles of TGF β /Activin/Nodal in the mouse placenta are better understood compared to the other member of this family, BMP. Recent studies in our lab suggest that BMP signaling is important for maintaining TS cells in an undifferentiated state (A. Wakeland and F. Soncin, unpublished). WT TS cells exhibit higher levels of phosphorylated Smad1/5/8 up to 24 hours after the initiation of differentiation, which decreases around 48 hours, suggesting that BMP signaling is active in the TS stem cell state. I confirmed that Sirt1-null cells express BMP receptors and BMP4 at levels comparable to WT trophoblasts; however, despite this, null TS cells have a dramatic decrease in BMP signaling through p-Smad1/5/8 and a decrease in total Smad1 protein. I confirmed this observation in two different WT and null TS cell lines. The dysregulation of BMP signaling in Sirt1-null TS cells was also supported by the microarray analysis: BMP signaling pathways comes up in gene ontology analysis of probes downregulated in null d0 cells.

A recent study suggested a link between Sirt1 and Smad1. In neuronal differentiation, this study found that in the absence of Syx, a gene encoding a RhoA-specific guanine nucleotide exchange factor (GEF), neuronal differentiation was enhanced, in part due to reduced p-Smad1 (Yang et al., 2016). These same Syx^{-/-} cells exhibited reduced Sirt1 protein levels, which increased with re-introduction of RhoA. A similar biological mechanism may contribute to the reduced p-Smad1/5/8 detected in undifferentiated Sirt1-null TS cells. Interestingly, in the microarray analysis of genes upregulated in null d0 cells, signaling by Rho Family GTPases does appear as one of the top canonical pathways potentially upregulated in the absence

of Sirt1. However, further studies are necessary to first understand the role of BMP signaling in mouse trophoblasts before we can fully determine the relationship between Sirt1 and BMP signaling in trophoblast stem cells.

Sirt1 may also, more widely, regulate the TGF β signaling superfamily through modulation of Smad4. In addition to reduced of BMP signaling, I also found that Sirt1-null TS cells do not respond to Activin A signaling at wild-type levels, as shown by reduced levels of p-Smad2/3. Though TGF β /Activin/Nodal and BMP have separate signaling cascades, Smad4 is known as the common denominator of both branches of TGF β signaling, interacting with both Smad1/5/8 and Smad 2/3 (Chaudhury and Howe, 2009). Studies have shown that Sirt1 can interact with and deacetylate Smad4 (Simic et al., 2013a; Chen et al., 2014; Xiao et al., 2016). Specifically in oral squamous cell carcinoma cells, (Chen et al., 2014) as well as breast cancer and kidney epithelial cells (Simic et al., 2013a), this Sirt1-Smad4 interaction inhibits MMP7 and downregulates epithelial-to-mesenchymal transitions. Therefore investigation of Smad4 in WT and Sirt1-null trophoblasts may elucidate an additional mechanism through which Sirt1 regulates TGF β signaling in the mouse placenta and trophoblast stem cells.

7.2 Sirt1-null trophoblasts are halted in an Epcam-positive labyrinthine progenitor trophoblast state due to HGF-cMet signaling

Despite the observation that Sirt1-null TS cells are more differentiated in their stem cell state compared to WT cells, TS cells lacking Sirt1 also exhibit differentiation defects. Decrease in Cdx2 and Esrrb during differentiation suggests that Sirt1-null TS cells differentiate; however, expression patterns of specific markers for all trophoblast lineages were consistently reduced in null trophoblasts instead of increasing similar to

WT trophoblasts. Both labyrinthine markers *Gcm1* and *SynA* were reduced, demonstrating reduced differentiation into this lineage. Similarly, differentiation into spongiotrophoblasts and TGC, as demonstrated by expression of *Tpbpa*, and *Prl3b1* and *Prl2c2*, respectively were reduced as well. PCA of gene expression profiling data demonstrated that null TS cells on d5-d7 do not align with WT d5-d7 cells but are instead closer to WT d2-d5. Together, these data clearly show that though TS cells differentiate in the absence of *Sirt1*, they do not differentiate into terminal trophoblast.

Instead trophoblast cells lacking *Sirt1* appear to be halted in a progenitor state. Though I tested for markers of both invasive and labyrinthine progenitor cells, only *Epcam*, a labyrinthine progenitor marker, was consistently maintained in differentiated *Sirt1*-null cells. Trophoblast cells expressing high levels of *Epcam* have been shown to give rise to all three labyrinthine lineages: S-TGC, SynT-I and SynT-II (Ueno et al., 2013). In addition to maintenance of *Epcam* RNA and protein expression, differentiated *Sirt1*-null trophoblast also had a higher proportion of such *Epcam*^{high} cells.

Though *Epcam*^{high} progenitor cells are incompletely understood, it is known that HGF-c-Met signaling is necessary to maintain this population of cells (Ueno et al., 2013). c-Met is a receptor tyrosine kinase (RTK) and hepatocyte growth factor (HGF) is its corresponding ligand (Organ and Tsao, 2011). HGF-null embryos die between e13.5 and e15.5 and null placentas have reduced trophoblast and defects in labyrinth vasculature development (Uehara et al., 1995). A more recent study discovered that c-Met signaling is needed for the proliferation of a population of *Epcam*^{high} labyrinthine progenitors both *in vivo* and *in vitro* (Ueno et al., 2013). Pathway analysis of genes upregulated in *Sirt1*-null d0 cells included HGF signaling as a possible enriched

pathway. Downstream of c-Met signaling are many different signaling cascades. Some of these, such as AKT and MAPK are also related to Sirt1 expression (Rafalski et al., 2013). However, I did not detect differences in activation of either of these pathways. Therefore, thus far, my data supports the hypothesis that in the absence of Sirt1, trophoblast exhibit dysregulation of c-Met signaling which halts the cells in an Epcam^{high} labyrinthine progenitor state.

How the absence of Sirt1 leads mechanistically to altered cMet signaling is unclear. There are no previous studies showing alteration of either HGF or c-Met downstream of Sirt1; nor has there been any evidence of c-Met protein regulation by acetylation status. Studies have, however, shown that c-Met RNA expression may be regulated by histone modification, specifically acetylation: inhibition of histone deacetylases (HDAC) in several cell types have led to increased c-Met RNA expression (Buurman et al., 2012; Sun et al., 2012; Zhang and Babic, 2016). However I only detected a significant increase in c-Met expression at the protein level.

I hypothesize that Sirt1-null trophoblast may instead fail to degrade c-Met protein. Of the genes downregulated in null TS cells, pathway analyses revealed protein ubiquitination as one such potentially enriched pathway. C-Met is known to be regulated by ubiquitination. Upon c-Met activation, c-Met is also phosphorylated at Y1003 to create a recruitment site for Cbl, an E3 ubiquitin ligase, needed for c-Met ubiquitination and degradation (Peschard et al., 2001). Socs1 also promotes degradation of RTKs such as c-Met and reduces phosphorylated c-Met in liver through a lysosome-independent mechanism (Thien et al., 2003). Evaluation of ubiquitination and degradation pathways in the Sirt1-null trophoblast may elucidate

defects in these cellular processes, leading to maintenance of a c-Met dependent, Epcam-positive progenitor cells in the absence of Sirt1.

7.3 Sirt1 regulates trophoblast proliferation and apoptosis

In addition to differentiation defects, TS cells lacking Sirt1 clearly demonstrate altered proliferation and apoptosis. During standard cell culture, Sirt1-null TS cells grew noticeably slower as indicated by reduced BrdU incorporation in the null TS cells, showing a decrease in the proportion of TS cells in S phase. Given that null TS cells have lost some level of stemness compared to WT counterparts, the reduced proliferation is a biologically consistent observation. Microarray analysis also highlighted genes involved in cell cycle regulation to be downregulated in null d0 cells.

Sirt1 is also a classic negative regulator of apoptosis, commonly through deacetylation of p53 (Luo et al., 2001; Vaziri et al., 2001). Preliminary experiments evaluating trophoblast apoptosis showed that Sirt1-null TS cells, when exposed to oxidative stress, exhibit a slight increase in apoptosis compared to wild-type TS cells. As expected, gene ontology shows the p53 pathway as the most statistically significant category of genes upregulated in null d0 cells. Sirt1 commonly plays a cytoprotective role and was originally studied as a regulator of cell survival upon calorie restriction in yeast (Cohen et al., 2004). Since then, Sirt1 has been shown to be a negative regulator of apoptosis in many systems including chondrocytes (Takayama et al., 2009) and pre-adipocytes (Chen et al., 2012). Sirt1 likely plays a role in trophoblast cytoprotection.

7.4 Sirt1 is important for *in vivo* placental development and differentiation into labyrinthine trophoblasts

Previous studies have consistently reported that homozygous Sirt1-null pups are growth restricted (Cheng et al., 2003; McBurney et al., 2003; Wang et al., 2008). Often, growth restriction can be a sign of placental defects. Though evaluations of these embryos and some developing tissues have been done, no previous study evaluated the placenta, or trophoblast, in the absence of Sirt1. I therefore used the Sirt1-null mice on the Sv129 genetic background to study Sirt1 *in vivo* in placental development.

We observed that, as previously reported, Sirt1-null embryos are consistently growth restricted, but we additionally discovered that null placentas have reduced weight. Further analysis of null placentas demonstrated an abnormal labyrinthine layer. The null labyrinth was hypercellular, at least in part due to an increase in clusters of Epcam-positive cells in the labyrinth. In WT placentas, Epcam-positive cells were maintained close to the fetal base of the placenta, while the null cells also showed such cells more centrally within the labyrinth. The null placentas also exhibited alterations in terminal labyrinthine lineages. Specifically, there is a defect in syncytiotrophoblast layer 1 and 2, as indicated by decrease in Gcm1 and an increase in SynA expression. Secondly, null placentas also have a smaller layer of Tpbpa positive spongiotrophoblast, indicative of defects in the junctional zone, though this was not due to a decrease in glycogen cells. However, there were no observable changes in the TGC *in vivo* despite reduced invasion of Sirt1-null trophoblast *in vitro*. Overall, I have identified both a labyrinthine and a junctional zone defect in placentas

lacking Sirt1. Placental specific knockdown of Sirt1 will confirm the contribution of placental defects to embryonic development and growth restriction.

The mouse labyrinth is similar to human placental villi, facilitating gas and nutrient exchange between the mother and developing fetus. The tri-chorial organization of mouse placentas is designed to regulate exchange between maternal and fetal blood. Given that there is a defect in SynT layers I and II, it is possible that alterations in Sirt1 lead to defects in nutrient exchange. Prior studies of Sirt1 during pregnancy and in the placenta suggest that Sirt1 acts as a nutrient sensor that reacts to maternal nutrients to affect embryo development. A high fat diet during gestation resulted in a decrease in placental Sirt1 and an increase in placental lipoprotein lipase, which facilitates fatty acid transport across the placenta (Qiao et al., 2015). Activation of Sirt1 also may facilitate Glut1-dependent glucose transport in placentas under oxidative stress (Lappas et al., 2012). It is possible that in Sirt1-null placentas, defects in the labyrinthine layers may result in dysregulation of known or novel nutrient transport across the placenta during pregnancy.

7.5 Role of Sirt1 activity in trophoblast differentiation and placental development

Generally, most Sirt1 function is attributed to its known deacetylase activity. However my attempts to characterize placental Sirt1 activity using Sirt1-mutant mouse and trophoblast stem cell models have not provided a clear conclusion. Sirt1-mutant mice did not display reduced growth of placentas or embryos, nor were placental defects observed upon further inspection of histology. Though I was able to derive trophoblast stem cell lines from Sirt1-mutant embryos, the isolated lines showed a premature differentiation defect, which was likely a technical issue with derivation, and not a true phenotype. The marker expression patterns of both Sirt1-

mutant lines were different in the undifferentiated state but both lines exhibited low levels of TS markers and higher expression of TGC markers. Further differentiation of these lines led to increased expression of spongiotrophoblast and TGC marker only. This is different from the Sirt1-null TS cells, in that, though these cells began more differentiated, they never exhibited WT levels of differentiation markers. A few studies in other cell models have suggested that Sirt1 may have a deacetylase-independent function (Ghosh et al., 2007; Jin et al., 2007; Pfister et al., 2008; Campagna et al., 2011); however, no specific mechanisms have yet been identified or characterized.

Though evaluation of Sirt1-mutant animals and cells were inconclusive, activation of Sirt1 using small molecules did modulate Sirt1 activity and affect cellular phenotype. In the presence of Resveratrol, a common Sirt1 activator, WT TS cells showed reduced junctional zone, but enhanced differentiation into the labyrinthine lineage, as demonstrated by an increase in SynA expression. Though resveratrol is known to have off target effects, the increase in lineage specific markers was Sirt1-specific because increase in labyrinthine differentiation occurred only in WT cells, and not in Sirt1-null trophoblast. Modulation of Sirt1 inhibitors, however, was not as clear. Sirtinol and Ex527, two common Sirt1 inhibitors, did not recapitulate the Sirt1-null trophoblast differentiation phenotype as would be expected if the phenotype was dependent on deacetylase activity. Furthermore, treatment of two WT cell lines with both Sirtinol and Ex527 did not consistently affect TS cell differentiation in a reproducible manner.

7.6 Future directions

Overall, my work has shown a novel role for Sirt1 in trophoblast differentiation and placental development, both *in vivo* and *in vitro*. Further work may elucidate the mechanisms by which Sirt1 acts through the BMP and c-Met signaling pathways to mediate these effects. However, since Sirt1 regulates many other cellular processes, other than differentiation, the role of Sirt1 in the placenta may reach beyond differentiation. This may include Sirt1 regulation of placental apoptosis or inflammation.

The role of Sirt1 in trophoblast cytoprotection would be useful to understand, as this would have implications for pregnancy disorders. Apoptosis is known to be important in human trophoblast turnover such as with the accumulation of syncytial knots of aging, dying nuclei found in placental villi which can shed into maternal circulation (Tomas et al., 2011). However this material may also contribute to placental disorders such as preeclampsia, a placenta related pregnancy complication characterized by proteinuria and hypertension (Cudmore et al., 2012). Preeclamptic placentas also exhibit an increase in trophoblast apoptosis (Allaire et al., 2000; Cudmore et al., 2012). Given the role of Sirt1 in cytoprotection in other tissues (Chen et al., 2009; Tang, 2009; Nath, 2010) and its known role as an anti-apoptotic regulator, Sirt1 may facilitate trophoblast protection.

Gene ontology analysis of genes upregulated in undifferentiated trophoblast stem cells in my microarray data set identified enrichment of genes involved in the p53 pathway, hypoxia and regulation of cellular response to stress. Sirt1 is a common p53 negative regulator (Vaziri et al., 2001) so enrichment of this pathway consistent with Sirt1 literature. It is possible that Sirt1, in trophoblasts, regulates p53 in response to cellular stress, such as hypoxia. Hypoxia is necessary during early placental

development, however shallow trophoblast invasion of the maternal decidua can result in pathologic hypoxia and can result in placental defects (Wu et al., 2015) and complications including preeclampsia. It is possible that this is partially regulated by p53. It has been previously shown that the p53 pathway is dysregulated in preeclampsia (Sharp et al., 2014).

Sirt1 is also a regulator of inflammation and has been implicated in playing a role in the inflammatory response during labor (Lappas et al., 2011; Kim et al., 2013). Of the canonical pathway and gene ontology analyses of genes up and downregulated in null TS cells in my microarray, many of these are involved in inflammatory response, including IL-6 and IL-8 signaling. Preeclampsia can result in maternal systemic inflammatory responses that may be caused by release of trophoblast microparticles, proteins or nucleic acids (Redman et al., 1999). Maternal serum levels of IL-6 and IL-8 can increase in severe preeclampsia cases (Sharp et al., 2014). It is possible that loss of Sirt1 results in increased levels of both in trophoblasts. Additionally, IFN- γ , which is also higher in preeclampsia, contributes to cytotrophoblasts migration in humans and regulating spiral artery remodeling in rodents but can also inhibit trophoblast invasion (Redman et al., 1999; Saito et al., 1999; Hu et al., 2006). Furthermore, preterm labor is marked by an increase in inflammation (Romero et al., 2006; Gotsch et al., 2009; Kemp, 2014). However pro-inflammatory signaling is important in labor initiation (Goldenberg et al., 2008; Kemp, 2014) and Sirt1 has already been implicated to potentially play a role in labor inflammation (Lappas et al., 2011; Tsai et al., 2015). Sirt1 may regulate placental inflammatory responses through IL-6, IL-8, IFN- γ or other pathways in both healthy and pathologic placentas. Confirming mechanisms by which Sirt1 regulates

inflammatory response of trophoblasts could lead to better understanding of placental inflammation and labor and how dysregulation of either contribute to preterm labor.

Finally, elucidating the role of small molecule activators and inhibitors of Sirt1 could similarly impact our understanding of pregnancy complications, potentially leading to improved treatments for placental disorders. Resveratrol has already been implicated in potentially being useful for preeclampsia, at least in an *ex vivo* model. Vascular endothelial growth factor receptor 1 (sFlt-1) is elevated in preeclampsia pregnancies (Vuorela et al., 2000; Maynard et al., 2003). It can result in angiogenesis inhibition (Ahmad and Ahmed, 2004) and studies in rodents demonstrate that it contributes to preeclampsia and its symptoms such as hypertension and proteinuria (Maynard et al., 2003; Bergmann et al., 2010). Resveratrol was able to reduce levels of sFlt-1 secretion from human placental villous explants from preeclamptic pregnancies as well from healthy explants treated with inflammatory factors (Cudmore et al., 2012). If Sirt1 does regulate apoptosis or inflammation in preeclampsia or other placental disorders, activation of Sirt1 may confer cytoprotection and help alleviate placental damage. Given the array of protein substrates and cellular processes regulated by Sirt1, future studies of placental Sirt1 and Sirt1 in trophoblasts may help progress understanding of both healthy and complicated pregnancies.

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