Investigating the physiological role of HDAC1 and HDAC2 in embryonic stem cells

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by

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Abstract

Histone deacetylases 1 and 2 (HDAC1/2) are highly similar proteins (83% identical) that form the core catalytic components of corepressor complexes that modulate gene expression. Germline deletion of *Hdac1* in mice results in early embryonic lethality and conditional deletion of *Hdac1* but not *Hdac2* causes precocious differentiation in ES cells. Therefore to further investigate the role of HDAC1/2 during the early embryogenesis, we have generated a compound conditional knockout ES cell line $Hdac1^{ko}$; $Hdac2^{Het}$ in which HDAC1/2 activity is reduced but not entirley lost. $Hdac1^{ko}$; $Hdac2^{He}$ cells have a significant reduction in total deacetylase activity and disruption of corepressor complex integrity.The prolifration capicity of $Hdac1^{ko}$; $Hdac2^{He}$ cells is not inhibited, however, upon differentiation they were predisposed to toward the cardiomyocyte lineage.

In most cell types, deletion of both *Hdac1* and *Hdac2* is required to produce a phenotype, suggesting their activity is redundant. To circumvent this functional redundancy, we generated a double conditional knockout (DKO) cells in which both *Hdac1* and *Hdac2* can be inactivated simultaneously. Loss of HDAC1/2 results in a 60% reduction in total HDAC activity and a loss of cell viability, which is associated with increased abnormal mitotic spindle, chromatin bridges and miconuclei, suggesting that HDAC1/2 are necessary for accurate chromosome segregation. Transcriptome analysis reveals 1,708 differentially expressed genes in DKO cells including a reduction in the expression of the ES cells core pluripotent factors. HDAC1/2 activity can be regulated in vitro through the binding of inositol tetraphosphate (IP4). By rescuing the viability of DKO cells using wt and mutant forms of HDAC1, we demonstrated that mutations that abolish IP4 binding reduce the activity of HDAC1/2 in vivo. We have also shown that treatment of DKO ES cells with RA results in reduces induction of HOX genes, suggesting a positive role of HDAC1/2 in gene activation as well as gene repression.

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Abbreviations:

4-OHT	4-hydroxytamoxifen
aa	amino acid
ac	acetyl
AP	alkaline phosphatase
ASCL1	achaete-scute homolog 1
ATP	adenosine triphosphate
BMP4	bone morphogenic protein4
BocK	Boc-acetyle lysine
Bp	base pair
BP-GO	biological process gene ontology
CaMK	calcium/calmodulin-dependent protein kinase
CDK	cyclin-dependent kinase inhibitors
cDNA	complimentary deoxyribonucleic acid
Cdx1	caudal type homeobox1
CHD	chromo-domain helicase DNA
CHD1-4	chromo-domain helicase DNA binding protein
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP combined with high-throughput sequencing
ChK1	checkpoint kinase1
cKO	conditional knock-out
CNS	central nervous system
Co-IP	coimmunoprecipitation
CoREST	co-repressor to REST
CreER	cre recombinase, estrogen receptor
Ct	cross threshold
CtBP	carboxyl-terminal terminal binding protein
CTT	carboxyl-terminal tail
CXCR4	C-X-C chemokine receptor type 4
CYP26a	cytochrome P450 26 subfamily
DAD	deacetylase activation domain
DAVID	database for annotation, visualization, and integrated discovery
DEPC	diethylpyrocarbonate
DMSO	dimethyle-sulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA (cytodine-5)-methyltransferase
Dnmt3b	DNA (cytodine-5)-methyltransferase 3b
E2F4	E2 transcription factor 4
EBs	embryoid bodies
eGFP	enhanced green fluorescent protein

ELM2	egl-27 and MTA1 homology 2 domain
ERK	extracellular signal-regulated kinase
Esrrb	estrogen-related receptor beta
FACS	fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
FBS	foetal bovine serum
Fc	fold change
FGF4	fibroblast growth factor 5
FRAP	fluorescent recovery after photobleach
FZD9	frizzled 9
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA4	GATA-binding protein4
GCNF	germ cell nuclear factor
GPS2	G protein suppressor 2
H	histone
HAT	histone acetyletransferases
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyle)-1-piperazineethanesulfonic acid
HEX	hexachlorofluorescein
HID	HDAC interaction domain
HOP	homeodomain only protein
HP1	heterochromatin protein 1
ICM	inner cell mas
Id	inhibitor of differentiation
Ikaros	Ikaros family zinc finger protein1
IL-10	interleukin 10
IP4	inositol tetraphosphate (1,4,5,6)
IVT	in vitro transcription
JMJD	jumonji C domain containing demethylase
KMT	lysine methyletransferase
LB	luria-bertani
LBD	ligand-binding domain
Lhx1	LIM homeobox1
LIF	leukemia inhibitory factor
LoxP	locus of X over P1
LSD1	lysine demethylase
MBD	methyl-CpG binding domain
me1,2,3	mono, di-, tri-methylation
MeCP	methyl-CpG binding protein 2
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor2
Mef2c	myocyte enhancer factor 2C
Meis2	homeobox protein Meis2

mES	mouse embryonic stem
Mi-2B	chromodomain helicase DNA binding protein3
MMP	matrix metalloproteinase
MTA1-3	metastasis associated protein
Myc	myelocytomatosis oncogene
MyoD	myogenic differentiation1
Nanog	nanog homeobox
NCoR	nuclear receptor corepressor
NKX2-5	homeobox protein NK-2 homolog E
NODE	Nanog- and Oct4-associated deacetylase complex
NuRD	nucleosome remodelling and histone deacetylase complex
Oct4	POU domain-containing transcription factor
Otx2	orthodenticle homeobox 2
P21	cyclin dependent kinase inhibitor 1A
P300	histone acetyletransferases p300
p53	tumor suppressor protein
P57	cyclin-dependent kinase inhibitor 1C
PAH	paired amphipathic helix
PARP	Poly (ADP-ribose) polymerase 1
PAX6	Paired box protein6
PBS	phosphate buffered saline
PBST	phosphate buffered saline- tween
PI	Propidium iodide
PKD	protein kinase D
PLB	protein loading buffer
PRC1	polycomb repressive complex1
PRC2	polycomb repressive complex2
PRMT	arginine methyletransferases
PS	phosphatidylserine
PTM	post-translation modification
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RbAp46/48	retinoblastoma associated protein
REST	repressor element-1 silencing transcription factor
Rif1	telomere-associated protein
RIN	RNA integrity number
Rme1	arginines can be mono-methylated
RNaseA	RNaseA
RT	room temperature
Runx2	runt-related transcription factor-2
RXR	retinoic X receptors

SAM	S-adenosylemethionine
SDS	sodium dodecyle sulphate
SDS-PAGE	sodium dodecyle sulphate polyacrylamide gel electropheresis
SDS3	suppressor of defective silencing 3
SEM	standard error of mean
SET	Su(var)3-9, Enhancer of Zeste and Trithorax
Sin3a	SWI-independent 3
Sir2	silent information regulator 2
SMRT	silencing mediator of retinoid and thyroid receptor
SOX1-2	SRY-Related HMG-Box
STAT3	signal transducer and activator transcription 3
Stra8	stimulated by retinoic acid 8
SUMO	small ubiquitin-related modifier
SWI/SNF	SWI/sucrose non-fermentable
TBL1	transducing β-like1)
TBX5	T-box transcription factor
TE	trophectoderm
TIMP1	inhibitor of metalloproteinase1
TNNT2	cardiac troponin type 2
TSA	trichostatin A
UBC9	SUMO-conjugating enzyme E2
UPL	universal probe library
WCE	whole cell extract
Wnt	wingless-integration 1
WT	wildtype

Chapter 1: Introduction

1.1 Chromatin

The genome of eukaryotic species is greatly compacted into chromatin, a dynamic protein-DNA structure that can change its shape and conformation during the life of a cell. The dynamic nature of chromatin plays a crucial role in regulating gene expression, structural and chemical modifications on chromatin can alter the expression of specific genes. Interphase chromatin exists in two forms based on its level of compaction: heterochromatin, a condensed form, which is transcriptionally inactive, and a less condensed/relaxed form, known as euchromatin that is transcriptionally active (Grewal, S., and Jia, S., 2007).

The basic repeating structural unit within eukaryotic chromatin is the nucleosome. In 1997, the crystal structure of the core nucleosome was determined by Timothy Richmond's group, it is composed of 146 base pairs (bp) of DNA wrapped 1.65 times around an octamer of histone proteins consisting of two molecules of each of the four core histones, in which two H2A-H2B dimers form a complex with an H3-H4 tetramer (Luger. K., et al., 1997). Each histone consists of a globular core domain and an unstructured N-terminal tail, which protrudes from the chromatin making it subject to covalent modifications which subsequently affect the level of chromatin compaction and alters the accessibility of DNA to the transcription machinery.

In the first level of chromatin compaction, the adjacent nucleosomes form nucleosomal arrays in an 10nm fiber known as “beads on a string” with approximately 20-80 bp of linker DNA between nucleosomal subunits which can be bound by histone 1 (H1) and other non-histone proteins (Zhou, YB., et al.,1998; Thoma, F., et al., 1979). Nucleosome units are further organized into a more compact structure known as the 30nm fiber with approximately six nucleosomes per turn in a helical solenoid or zigzag model (Finch, J., and Klug, A., 1976; Thoma, F., et al., 1979) (Figure 1.1). During metaphase, the chromatin is further compacted into loops with the assistance of fibrous proteins to generate a highly condensed chromatin.

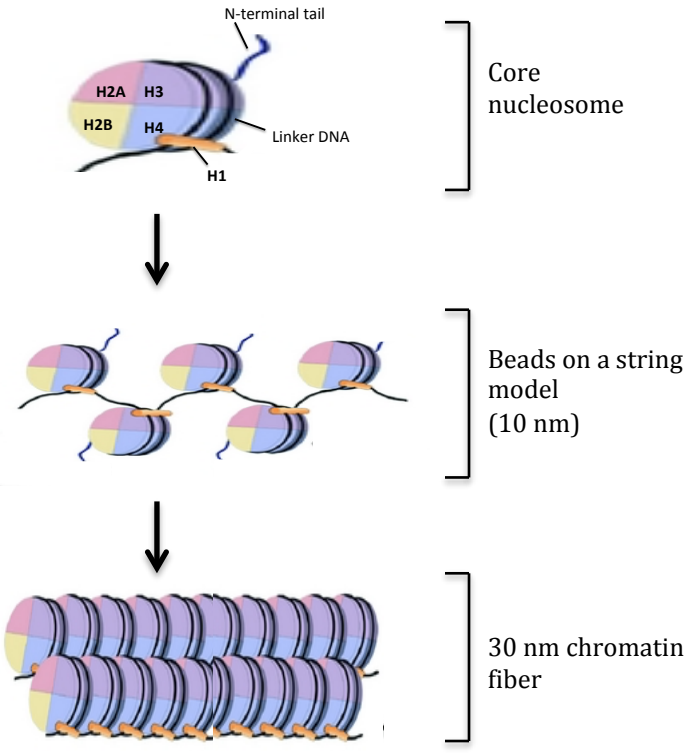


Figure 1.1: Phases of chromatin compaction. DNA wrapped 1.65 times around an octamer of histone which then further compact to form a 30nm chromatin fiber.

As described above, the level of chromatin compaction can affect the ability of gene transcription machinery to access DNA and thereby control gene expression. There are a number of mechanisms that are able to modulate chromatin structure, including: ATP-dependent remodeling complexes, DNA methylation (CpG di-nucleotide methylation) and post-translation modification of histones.

ATP-dependent chromatin remodeling complexes utilize the energy from the hydrolysis of ATP to reposition nucleosomes. They are large group of proteins conserved within eukaryotes, characterized by the presence of the ATPase subunit of SNF2 and classified into four different families: SWI/SNF family that is required for gene activation, ISWI or imitation SWI family which are involved in transcription repression, Mi-2 or CHD family that possess deacetylase activity in addition to their chromatin remodeling, and INO80 family which are involved in various biological processes, including transcription, DNA replication and DNA repair (Clapier, C., et al., 2009; Jin, J., et al., 2005).

DNA methylation is a common epigenetic modification associated with gene silencing and is important for the regulation of development and a number of key processes including genomic imprinting. DNA methylation occurs via DNA methyletransferases (DNMTs), which attach a methyl group to the 5-position of cytosine within the context of CpG dinucleotides. A CpG refers to a dinucleotide of cytosine and guanine bases that are connected by phosphodiester bond, the unmethylated CpG sites are often clustered together in CpG islands which are 1000-2000 bp in length and found in the vicinity of gene promoters. Methylated DNA is bound by methyl-CpG binding proteins (MBDs), which recruit other protein complexes, causing chromatin compacting and transcriptional repression. For example, MeCP2 (methyl-CpG binding protein 2) is a

member of MBD family that interacts with transcriptional corepressor complex Sin3a (Cukier, H., et al., 2008). Moreover, MBD2 (methyl-CpG binding domain2) is associated with NuRD corepressor complex that repress the transcription through the deacetylation of histone tails by HDACs (Hendrich, B., et al., 2001).

Remodeling of chromatin structure can also be achieved by covalently modifying histones, which effect the chromatin compaction and thereby influence gene expression. Most histone modifications predominantly occur at their unstructured N-terminal tails. Important histone modifications include acetylation, methylation, phosphorylation and ubiquitylation.

1.2 Histone modifications

The flexible charged N-terminal tails of histones protrude from the nucleosome and are subject to a several types of post-translation modifications (PTMs) that were first identified in the 1960s. Allfrey et al., showed that histone acetylation correlated with the level of RNA synthesis and regulation (Allfrey, V., et al, 1964). Histone modifications may directly alter chromatin compaction by influencing nucleosomes interactions or they can act as marks to be recognized by other non-histone protein complexes. More than 60 different histone residues have been found to be modified to date, with eight distinct types of modifications, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, deamination and proline isomeration (Kouzarides. T., 2007). Most of the enzymes that carry out these

modifications are involved in regulating gene expression or other genomic functions. Table 1.1 summarizes a number of posttranslation modifications of core histones.

The combination of modification marks on histones and their biological significance led to the proposal of a " histone code hypothesis" in which the pattern of histone modifications acts as a code that can be recognized (or read) by protein complexes that modulate gene expression through their effect on chromatin structure (Allis, C.D. and Strahl, B.D, 2000). Different types of histone modifications cooperate or "cross-talk" to regulate biological processes. PTM cross-talk can serve as a signal that promotes or blocks the addition of a second modification. Cross-talk between modifications can occur on a single histone or between histones in a single nucleosome, or across nucleosomes (Suganuma, T., et al. 2008; Fischle, W., et al. 2003).

PTM	Histone	Residue	Transcriptional role or/Function
Acetylation	H3 H4 H2A H2B	K4, 9, 14, 18, 36, 56 K 5, 8, 12,16 K5 K5, 12, 15, 20	Activation Activation Activation Activation
Phosphorylation	H3 H4 H2A	T3, T11, S10 S28 S1 S1, T120	Mitosis Activation Activation Mitosis
Methylation	H3 H4 	R2, 8,17,26 K4, 36 K9, 27 K20 R3	Activation Activation Repression Repression Activation
Ubiquitination	H2A H2B	K119 K120	Repression Activation
Sumoylation	H2B H2A H4	K6, 7 K126	Repression Repression Repression
Isomeration	H3	P30 P38	Activation Repression

Table 1.1 Post-translation modification of histone tails. K, lysine; T, threonine; S, serine; R, arginine; P, proline. (Adapted from Berger, S.L., 2007 and Kouzarides, T., 2007).

There are distinct sets of histone modifications associated with silent heterochromatin, which generally shows low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation, whereas the actively transcribed euchromatin shows high levels of acetylation and tri-methylated H3K4, H3K36, and H3K79. Below, I will discuss how each of the individual types of histone PTMs effects chromatin structure and gene expression.

1.2.1 Acetylation

The first histone PTMs shown to have an effect on chromatin compaction and associated with transcription activation was acetylation (Allfrey, V., et al, 1964; Hebbes, T., et al., 1988). Histone acetyletransferases (HATs) catalyze the addition of acetyl group to the ε-amino group of lysine residues on the N-terminal tails of histones which neutralize the positively charged nitrogen atom that mediates the interaction between histone tails and negatively charged DNA and thereby increasing chromatin accessibility. HATs can be divided into two major classes: Type-A HATs, catalyze the acetylation of nucleosomal histones in the nucleus, and Type-B HATs, catalyze the acetylation of the newly synthesized histones (H4 at K5 and K12 and different sites on H3) in the cytoplasm leading to their transport to the nucleus and deposition onto newly replicated DNA (Sterner, D., and Berger, S., 2000). Three families of HATs have been identified based on their catalytic domains: GANT, MYST and CBP/p300 (Verdone , L., et al., 2006). Most acetylation sites are present within the N-terminal tails of histones H3 (K4, 9, 14, 18, 36) and H4 (K5, 8, 12, 16), which are positively correlated with gene activation. The steady-state level of lysine acetylation and deacetylation is achieved through the action of histone acetyletransferases (HATs) and histone deacetylases (HDACs). Deacetylation restores the positively charge to histones and thus yields a compact chromatin structure and consequently represses gene transcription (discussed in greater details in 1.3) (Figure 1.2).

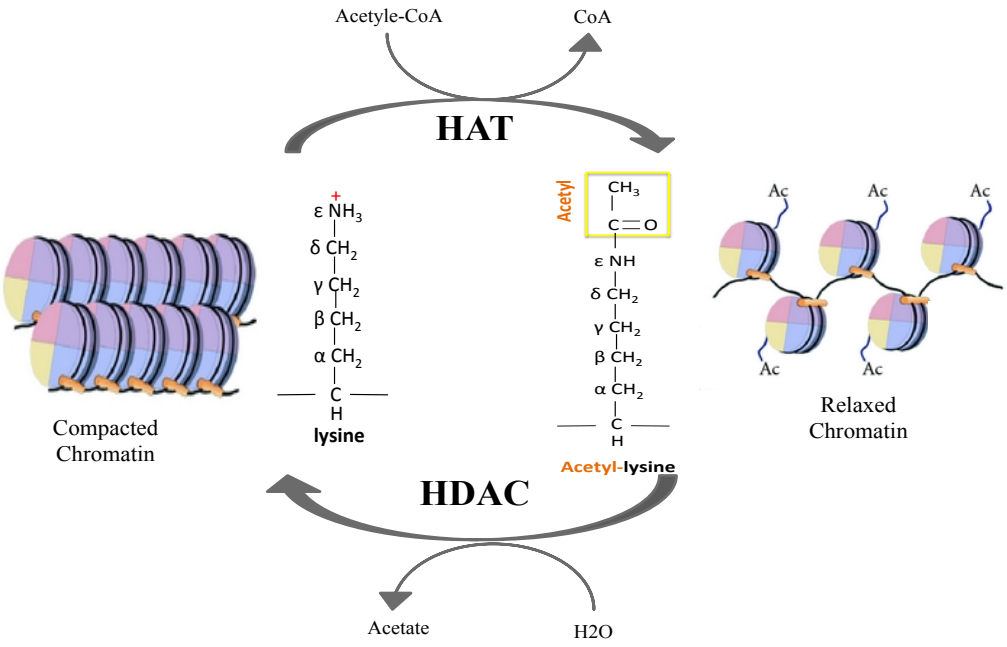


Figure 1.2: The reversible role of histone acetyletransferases (HATs) and histone deacetylases (HDACs) on chromatin compaction. (Rodd, A.L., et al., 2012).

1.2.2 Phosphorylation

Histone phosphorylation is also associated with transcriptional activation. Histones are phosphorylated at serines, tyrosines and threonines by specific protein kinases which catalyzed the addition of a phosphate group (PO4) while phosphatase mediate removal of it. Phosphorylation of serine 10 of histone H3 (H3S10P) is the most studied site and associated with transcriptional activation of genes, for instance heat shock genes. However, the H3S10P has been also involved in chromosome condensation and segregation during mitosis (Nowak, S., 2004), which indicates that the effect of this modification is more context-dependent. Phosphorylation of Threonine 11 of Histone 3

(H3Thr11P) by Dlk/Zip kinase is predominant at the centromeres during mitosis. It can be further phosphorylated by ChK1 and has been involved in transcriptional repression of specific genes upon DNA damage (Banerjee, T., and Chakravarti, D., 2011).

1.2.3 Ubiquitylation and sumoylation

Histones are also subjected to ubiquitylation, which refers to the covalent attachment of a 76 amino acid protein (ubiquitin) to the ε-amino group of lysine residues, and sumoylation, which is the addition of SUMO (small ubiquitin-related modifier) protein to the lysine residue. Histone H2A and H2B are the most highly ubiquitinatd sites, they can be mono- or polyubiquitylated, however, the most abundant forms is monoubiquitylated of H2A on lysine119 and H2B on lysine 120. The ubiquitylation of H2A at lysine 119 that mediated by Ring1b (E3 ligase) is associated with transcriptional repression. Ring1b was found in polycomb repressive complex 1(PRC1) which plays a role in gene silencing (Cao, J. and Yan, Q., 2012). Contrary to H2A, The ubiquitylation of H2B at lysine 120 is correlated with gene activation, for instance, HOX gene expression (Zhu, B., et al., 2005). Histone sumoylation has been found on H2A, H2B and mainly on H4 (Nathan, D., et al., 2006). Sumoylation of Histone H4 by UBC9 (SUMO-conjugating enzyme E2) is associated with transcriptional repression through the recruitment of histone deacetylases (HDACs) and heterochromatin protein1 (HP1) (Shiio, Y., et al., 2003).

1.2.4 Methylation

Unlike the other histone modifications discussed so far, histone methylation can occur at lysine or arginine residues and does not alter the positive charge of the amino acids. Lysines can be mono-methylated (me1), di-methylated (me2) or tri-methylated (me3), whereas arginines can be mono-methylated (Rme1), symmetrically (Rme2s) or asymmetrically (Rme2a) di-methylated. Lysine methyletransferases (KMTs) are responsible for the addition of a methyl group from the donor S-adenosylemethionine (SAM) to ε-amino group on lysine residue. With the exception DOT-1, all lysine known methyletransferases contain a conserved SET domain (Su(var)3-9, Enhancer of Zeste and Trithorax) responsible for the enzymatic activity (Rea, S., et al., 2000). In contrast to histone acetyletransferases **(**HATs), KMTs show a high specificity for their target lysine and the degree of methylation. For example, EZH2 KMT (a part of PRC2 complex) catalyzes H3K27me3, whereas, Suv39h KMT is responsible for methylation of H3K9me3 which is associated with formation of heterochromatin (O'Carroll,D., et al., 2001; Lachner, M., et al., 2001). Arginine is methylated by a distinct group of methyletransferases known as protein arginine methyletransferases (PRMTs), which catalyze the transfer of methyl group from SAM to the ω-guanidino group of arginine. PRMTs are classified into two classes, type I (Rme1 and Rme2as) and type II (Rme1 and Rem2s). Originally, the methylation of lysine was proposed to be stable and irreversible until the discovery of the first lysine demethylase, LSD1, which mediates demethylation of H3K4me2 (Shi., Y., et al., 2004). Lysine demethylases are now classified into two families; amine oxidases (LSD1 and LSD2) and Jumonji C (Jmjc-) domain containing demethylase (JMJD) (Whetstine., J., et al., 2006 and Tsukada., Y.,

et al., 2006). Lysine methylations are associated with transcriptional activation (H3K4, H3K36 and H3K79) and repression (H3K9, H3K27 and H4K20). The effect of Lysine methylation on gene transcription is context dependent, relying on the specific residue and the number of methyl moieties. For example, transcriptional activation is associated with methylation of H3K4, H3K36 and H3K79; while repression is linked with the methylation of H3K9, H3K27 and H4K20. The functionality of these sites is manifested by the recruitment of a specific binding protein with cognate chromodomain, tudor domain or PhD fingers. For example, the chromodomain containing heterochromatin protein 1 (HP1) binds H3K9me3 and is responsible for establishing constitutive heterochromatin and gene silencing (Bannister, A., et al., 2001). H3K27me3 recruits the PRC1 complex (polycomb repressive complex1) which is implicated in silencing HOX genes and X chromosome inactivation and genomic imprinting (Bracken., A., et al.,2006). On the other hand, the ATP-dependent chromatin remodelers CHD1 binds H3H3K4me3 through their chromodomain, this modification is predominantly present around the transcription start site and associated with transcriptional activation (Barski., A., et al.,2007).

1.3 The Histone deacetylases (HDACs) family

The first HDAC (HDAC1) was identified in 1996 using the HDAC inhibitor trapoxin as an affinity tag (Taunton, J., et al., 1996), and was found to be an orthologue of the yeast protein, Rpd3 which was known to be a global gene regulator with histone deacetylase activity (Vidal, M. and Gaber, R., 1991). Subsequently, using a combination of protein homology and complex purification, 18 mammalian HDACs have been identified and classified based on their homology to the yeast HDAC Rpd3. These HDACs have been designated as the Zn^{+2} dependent "classical family ", since the discovery of Sir2 (silent information regulator 2) or sirtuin protein family that are NAD^{+} dependent (Haigis, M. and Guarente. L., 2006) . The classical HDAC family is grouped into class I, class II and class IV, with class II being further subdivided into subclasses IIa and IIb (Figure 1.3). The NAD^{+} dependent sirtuins were referred as class III (De Ruijter. A., et al., 2003). The HDAC classes are different in their structure, function, subcellular localization and expression patterns. I will discuss each of the different HDAC classes below.

1.3.1 Four distinct HDAC classes

The class I HDAC family consists of HDAC1, HDAC2, HDAC3 and HDAC8, which are ubiquitously expressed, localized in the nucleus and exhibit high enzymatic activity toward histone substrates (De Ruijter. A., et al., 2003). With the exception of HDAC8, the class I HDACs are components of multiprotein complexes, which are crucial for

their transcriptional repression activity. HDAC1 and HDAC2 are highly similar proteins (83% sequence identity), with conserved catalytic domains and divergent C-terminal tails, which harbor two tandem casein kinase 2 phosphorylation sites that can be modified and effect their deacetylase activity and complex formation (Sengupta, N., and Seto, E., 2004). In mammalian cells, HDAC1 and 2 are found together in three main co-repressor complexes which modulate their deacetylase activity and DNA binding. The main HDAC1/2 containing complexes are the Sin3A, NuRD and CoREST (Yang, X. and Seto, E., 2008), discussed in detail below (section 1.4). HDAC3 shares 68% sequence identity with HDAC1and HDAC2, although it exits in distinct co-repressor complexes with the nuclear receptor co-repressor SMRT and NCoR, which are essential for activation of its deacetylase activity (Watson, P., et al., 2012; Millard, C., et al., 2013). HDAC8 is most similar to HDAC3 (34% identical) and it is fully functional in solution without need to interact with a co-repressor complex.

Class IIa HDACs comprise HDAC4, HDAC5, HDAC7 and HDAC9, which consists of deacetylase domain and a conserved long N-terminal region that contain conserved binding sites for the transcription factor MEF2 (myocyte enhancer factor2) and the chaperone protein 14-3-3. Upon phosphorylation by kinases, for instance CaMK (calcium/calmodulin-dependent protein kinases) or PKD (protein kinase D), these HDACs shuttle from the nucleus to the cytoplasm through their binding with 14-3-3 protein (Grozinger, C., and Schreiber, S., 2000). In contrast, association with MEF2 promotes nuclear localization of HDACs and therefore leads to transcriptional repression of the target genes. Class IIa HDACs have negligble deacetylase activity due to the presence of Histidine rather than Tyrosine in their catalytic site (Lahm. A., et al., 2007). In contrast to class I HDACs, the expression and function of class IIa is

tissue-specific, HDAC4, 5 and 9 are enriched in brain heart and muscle, whereas, HDAC7 is highly expressed in endothelial cells and thymocytes (Heberland, M., et al., 2009b and De Ruijter. A., et al., 2003).

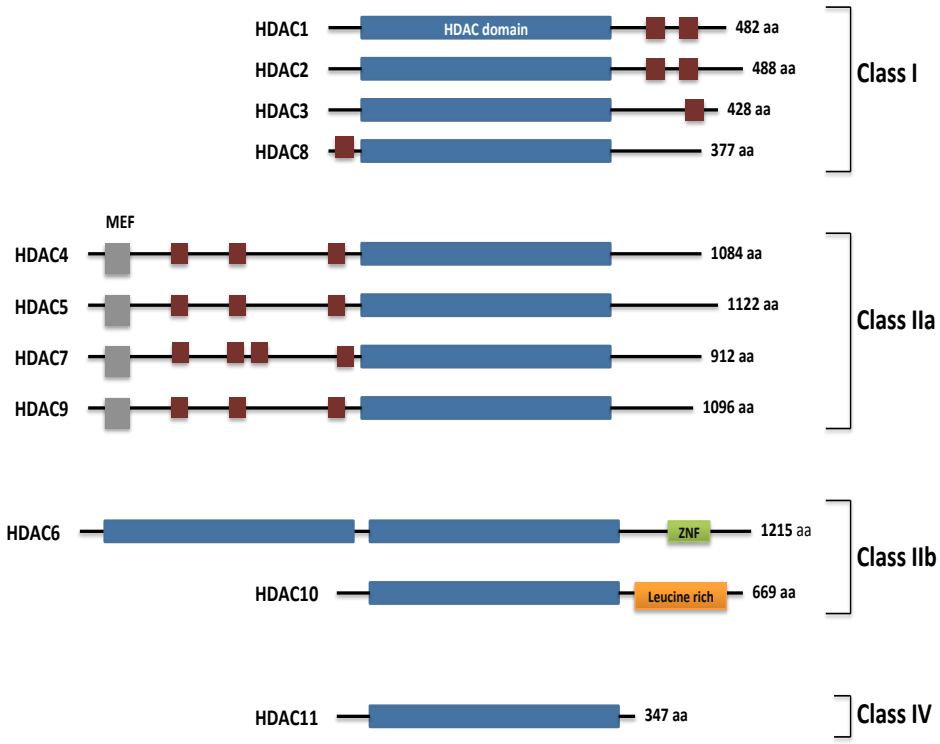


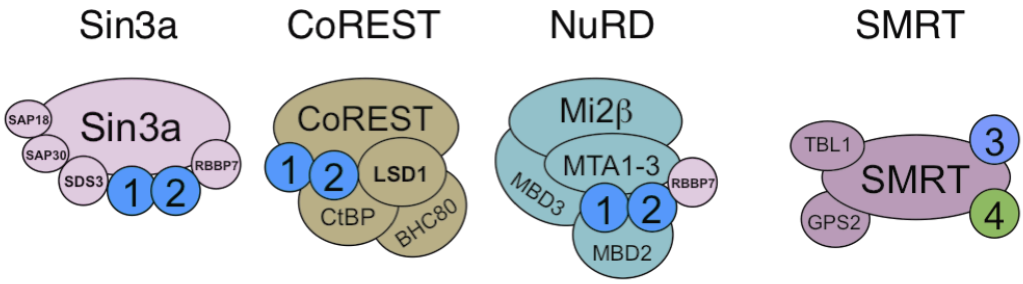
Figure 1.3: Classification and domain organization of the classical (Zn2+ dependent) histone deacetylases (HDAC) family. Dark blue bars represent the deacetylase domain, red bars represent serine phosphorylation sites and grey bars represent Myocyte enhancer factor 2(MEF)-binding motifs (Adapted from Mihaylova and Shaw, 2013).

Class IIb contains only HDAC6 and HDAC10. HDAC6 is the main cytoplasmic deacetylase enzyme and it is distinct from all other HDACs, as it contains two tandem deacetylase domains and C-terminal zinc finger domain that can bind ubiquitin (Yang. X., and Seto. X., 2008). HDAC10 has one deacetylase domain that is highly similar to the N-terminal deacetylase domain of the HDAC6, and a leucine-rich C-terminal domain. HDAC10 plays a role in suppression of cervical cancer through inhibition of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) (Song, C., et al., 2013).

HDAC11 is the sole member of class IV HDACs. HDAC11 is highly conserved across species, composed of a deacetylase domain that is closely related to class I and class II HDACs, and a small N-terminal domain (Gao, L., et al., 2002). HDAC11 has been shown to negatively regulate interleukin 10 (IL-10), which effects the inflammatory response (Villagra, A., et al., 2009).

1.4 Class I HDAC co-repressor complexes

With the exception of HDAC8, all class I HDACs must be recruited into multiprotein corepressor complexes to activate their enzymatic activity and be recruited to their appropriate genomic targets. The main co-repressor complexes for Class I HDACs in mammalian cells are Sin3A, NuRD, CoREST and NCoR/SMRT (Figure 1.4). HDAC1 and HDAC2 form the catalytic core of all co-repressor complexes, except HDAC3 in the NCoR/SMRT complex.



Complex	Component	Protein domain
Sin3	HDAC1, HDAC2	deacetylase
	RbAp46, RbAp46	WD40 repeat
	Sin3A	PAH motifs
	Sds3	
	RBP1	
	SAP18	Ubiquitin fold
	SAP30	
	ING1/2	PHD finger
NuRD	HDAC1,HDAC2	Deacetylase
	RbAp46, RbAp46	WD40 repeat
	Mi2α/β	Helicase
	MTA1/2/3	SANT domain
	MBD2/3	Methyl CpG binding
	P66α/β	
CoREST	HDAC1,HDAC2	Deacetylase
	CoREST	SANT domain
	LSD1	SWIRM domain
	BCH30	PHD finger
	CtBP	Dehydrogenase
NCoR/SMRT	HDAC3, HDAC4	Deacetylase
	NCoR/SMRT	SANT domain
	TBL1/TBLR1	WD40 repeat
	GPS2	
	JMJD2A	PHD finger/Tudor domain
	Kaiso	Methyl CpG binding

Figure1.4: Class I HDAC co-repressor complexes. The schematic shows composition co-repressor complexes (kindly provided by Dr.Shaun Cowley). The table detailed the list of components and protein binding domains (Adapted from Yang, X. and Seto, E., 2008).

1.4.1 Sin3 complex

Sin3 was initially identified as the corepressor utilized by Mad-Max in order to repress Myc target genes. Sin3 was identified as a homolog of the yeast transcriptional repressor Sin3 (Ayer. D., et al., 1995). Mammals have two Sin3 isoforms, Sin3A and Sin3B, which are 57% identical sharing highly conserved PAH and HID (HDAC interaction domain) domains. Sin3A and B are thought to provide a platform for the assembly of the complex, while HDAC1/2 provides the catalytic activity. SDS3 (suppressor of defective silencing 3) is an integral component that is required for the integrity and catalytic activity of the complex. The RbAp46/48 (retinoblastoma associated protein) are important to stabilise the interaction with the nucleosome. The Sin3A complex does not contain a DNA binding motif; therefore, it is recruited to chromatin targets by interacting with DNA-binding transcription factors, such as Mad1, Ikaros and p53 (Silverstein, R., and Ekwall, K., 2005). Sin3A is required for early embryonic development and T-cell proliferation. Knockout of mSin3A in mouse embryonic fibroblasts (MEFs) results in de-repression of genes involved in cell cycle progression, apoptosis, DNA replication, DNA repair and delocalization of HP1α (heterochromatic protein) (Cowley et al., 2005; Dannenberg et al., 2005). Sin3B plays an essential role in late stage of development in mice, deletion of Sin3B shows defect of multiple lineages differentiation due to de-repression of E2F4 and Mxd1 target genes (David et al. 2008).

1.4.2 CoREST complex

CoREST was initially identified as a corepressor of REST (repressor element-1 silencing transcription factor), which plays an important role in the regulation expression of neuronal genes in non-neuronal cells (Andres, M., et al 1999). Subsequently, CoREST was demonstrated by You et al., to be a component of HDAC1/2-containing co-repressor complex (You, A., et al 2001). HDAC1 and HDAC2 interact with the ELM2/SANT1 domains of CoREST, which confers catalytic activity within the CoREST complex. Further components include, LSD1 (Lysine specific demethylase 1) which demethylates H3K4me2/me (a positive marker of transcription) and regulates the stability of the CoREST complex (Foster, C., et al., 2010). The transcriptional corepressor CtBP (C-terminal binding protein) is also a member of the CoREST complex (Hayakawa, T., and Nakayama, T., 2011). Through its recruitment by REST, the CoREST complex is involved in regulating neural gene expression by deacetylation and demethylation of histones of histone tails (Lakowski, B., et al 2006).

1.4.3 NuRD complex

NuRD (nucleosome remodelling and histone deacetylation) complex was initially characterized based on its chromatin remodelling and deacetylase activities (Xue, Y., et al., 1998). The catalytic core of the NuRD consists of HDAC1, HDAC2, RbAp46, RbAp48 and metastasis associated protein (MTA) isoforms1, 2, and 3. All three MTA proteins contain an ELM2-SANT domain which directly recruits and activates

HDAC1 in the presence of inositol phosphate (IP4) (Millard, C., et al., 2013). The chromatin remodelling activity of NuRD is provided by CHD3/CHD4 (Mi-2α/β) which are members of SWI2/SNF2 chromatin-remodelling ATPase family. NuRD is integrated into epigenetic gene regulation further by the presence of MBD2 and MBD3, which belong to methyl-CpG binding domain (MBD) family. Although a central component of NuRD, MBD3 is unable to bind methylated-CpG, however, it is essential for mouse development (Hendrich et al., 2001). Embryonic stem (ES) cells lacking MBD3 (which disrupts NuRD) show a defect in differentiation due to the inability to repress OCT4. (Kaji, K., et al., 2006). A unique NuRD-like complex has been identified in ES cells, which contains Oct4, Nanog and the core components in NuRD complex but lacks MBD3. This complex named NODE (Nanog- and Oct4-associated deacetylase), displays deacetylase activity comparable to that of NuRD complex (Liang, J., et al., 2008). It has shown that, knockdown of NODE components leads to increased expression of differentiation genes.

1.4.4 SMRT/NCoR complex

The silencing mediator of retinoid and thyroid receptor (SMRT or NCOR2) and nuclear receptor corepressor (NCoR or NCOR1) are homologous proteins that share 40% identity. HDAC3 is the catalytic component of the complexes; its activity depends on the interaction with a conserved DAD (deacetylase activation domain) within SMRT in combination with IP4 (Watson, P., et al., 2012). The NCoR/SMRT complexes also contain TBL1 (transducing β-like1) that interacts directly with chromatin and mediates the function of HDAC3, and GPS2 (G protein suppressor 2)

that stabilises the assembly of the complex (Wong, M., et al., 2014). NCoR /SMRT are important for development, NCoR is essential for neural differentiation and T-cell development (Jepsen, K., et al., 2000), whereas SMRT is essential for heart development (Jepsen, K., et al., 2007).

1.5 Role of HDACs in transcriptional activation

A correlation between histone acetylation and increased gene expression was established earlier, in which acetylation of lysine residues within histone tails induced relaxation of chromatin structure. According to this model, histone acetyltransferases (HATs) are associated with transcriptional activation whereas histone deacetylases (HDACs) are associated with transcriptional repression. However, the transcription profiles of the yeast deleted for Rpd3, the yeast orthologue of HDAC1, revealed that the number of transcripts that were down-regulated was more than up-regulated (Bernstein, B., et al., 2000). Treatment of yeast with Trichostatin A (TSA), a class I and II HDAC inhibitor, also results in down-regulation of certain genes within 15 minutes of treatment, suggesting a function for HDACs in transcriptional activation.

Other studies have also suggested a link between HDACs and transcriptional activation. Kurdistani et al., used ChIP (chromatin immunoprecipitation) to map the genome-wide binding sites of Rpd3 in yeast, and found that it was preferentially associated with regions upstream of active genes (Kurdistani, S., et al., 2002) Moreover, in human primary $CD4^+$ T cells, ChIP-seq (ChIP combined with high-

throughput sequencing) experiments revealed an enrichment of HDACs (class I and class II) at transcriptionally active and primed genes, which co-localised with many HATs (Wang, Z., et al., 2009). In embryonic stem (ES) cells, HDAC1 was found to predominantly bind active genes including pluripotency factors, *Oct4*, *Nanog* and *Sox2* (Kidder, B., et al., 2011). The recruitment of HDACs to active genes is thought to reset chromatin state after the actions of HATs and RNA polymerase II, suggesting that gene activation process requires a cyclical utilization of both HATs and HDACs (Wang, Z., et al., 2009). Therefore one model which explains these observations is that HDACs participate in promoter clearance required to reinitialize the promoter for multiple rounds of transcriptional activation (Dovey, O., et al., 2010).

1.6 Non-Histone target of HDACs

Histone deacetylases (HDACs) are generally identified with the deacetylation of lysine residues within the histone proteins. However, a number of non-histone proteins have also been shown to be deacetylated by HDACs, including the transcription factors p53, E2F, STAT3 and GATA4 (GATA-binding protein4) (Gu, W. and Roeder, R., 1997; Boyes, J., et al., 1998). Moreover, HDAC6 regulates microtubule cell motility by deacetylation of α-tubulin, cytoskeletal protein (Hubbert, C., et al., 2002). A mass-spectrometry based analysis of the "acetylome" of three independent cell lines identified 3,600 lysine acetylation sites on 1,750 proteins involved in major molecular process including chromatin remodelling, transcription, DNA replication, cell cycle

and splicing (Choudhary, C., et al., 2009), suggesting that acetylation of lysine is an abundant post-translation modification which contributes to many cellular processes.

1.7 HDAC knock-out mice.

Deletion of each member of the class I HDACs leads to lethality in mice, suggesting an essential role of each HDAC (Table 1.2). HDAC1 has an essential role during embryogenesis. HDAC1-null mice die before embryonic day 10.5 and exhibit severe proliferation defects and growth retardation (Lagger, G., et al., 2002). In contrast, HDAC2 knock-out mice exhibit a phenotype in late embryos and adult animals. In one study, HDAC2-null (*Hdac2*$^{-/-}$) mice die within the first 24 hours after birth due to cardiac defect associated with uncontrolled proliferation of cardiomyocytes that leads to obliteration of the right ventricle lumen (Montgomery, R., et al., 2007). However, in two further studies (using the same genetrap constructs) nearly half of the *Hdac2*$^{-/-}$ pups died during the first 25 postnatal days, whereas the remaining littermates survived. The surviving mice had smaller heart than wild-type littermates and were unable to show normal cardiac hypertrophic responses (Trivedi, C., et al., 2007), and a decreased in the incidence of intestinal tumour formation (Zimmermann, S., et al., 2007). The cardiac defects have been previously linked to the disruption of HOP (homeodomain only protein) which functions during cardiac development as a regulator of cardiomyocyte proliferation (Chen, F., et al., 2002). It has been found previously that HOP interacts with HDAC2, deletion of HOP results in hyperprolifration of cardiomyocytes, suggesting that HOP-HDAC2 co-repressive interactions regulate cardiac proliferation and differentiation. Combinatorial and tissue

specific deletion of HDAC1/2 will be discussed in more detail below (section 1.8).

HDAC3 is also required for early embryonic development. HDAC3-null (*Hdac3*$^{-/-}$) mice die before embryonic day 9.5 (E9.5) due to gastrulation defects (Montgomery, R., et al., 2008; Bhaskara, S.et al., 2008). Deletion of HDAC8 in mice results in perinatal lethality due to craniofacial abnormalities, this result was phenocopied upon conditional deletion in neural crest cells as a result of the de-repression of homeobox transcription factors such as Otx2 and Lhx1 (Haberland, M., et al., 2009a).

Several of the Class II HDACs have also been deleted in mouse models. HDAC4 has an essential role in the skeleton formation; it is expressed in the chondrocyte hypertrophy during endochondral ossification. HDAC4 negatively regulates Runx2 (runt-related transcription factor-2), which regulates the development of bone. Therefore loss of HDAC4 leads to excessive bone formation such that the rib-cage cannot expand and the mice can not breath properly and die by postnatal day 10 (P10) (Vega, R., et al., 2004). Mice lacking HDAC5 or HDAC9 are viable, whereas compound mutants HDAC5/HDAC9 die during embryogenesis and the perinatal period due to defects in growth and maturation of cardiomyocytes (Chang, S., et al., 2004). Loss of HDAC5/9 deregulates MEF2 (myocyte enhancer factor-2) activity resulting in precocious differentiation of cardiomyocytes and cardiac defects. In addition, double mutant mice are hypersensitive to cardiac stress (Chang, S., et al., 2004). HDAC7-null mice die by day (E11.0) due to cardiovascular defects. Deletion of HDAC7 leads to up-regulation of MEF-2 target gene, MMP10 (matrix metalloproteinase 10), combined with down-regulation of TIMP1 (inhibitor of metalloproteinase) leading to dilatation and ruptured blood vessels (Chang, S., et al.,

2006). In agreement with the function of HDAC6 as the main tubulin deacetylase, HDAC6-null mice are viable and display a massive increased in acetylated α-tubulin (Zhang, Y., et al.,2008).

Class	Member	Phenotype of mouse gene deletion
I	HDAC1	Embryonic lethality by E10.5 due to proliferation defects
	HDAC2	Perinatal death due to cardiac defect
	HDAC3	Embryonic lethality by E9.5 with gastrulation defects
	HDAC8	Perinatal lethality due to craniofacial abnormalities
IIa	HDAC4	Postnatal death (P10), chondrocyte hypertrophy
	HDAC5	Cardiac hypertrophy
	HDAC9	Cardiac hypertrophy
	HDAC7	Embryonic lethality E11 due to cardiovascular defects
IIb	HDAC6	Increased in acetylated α-tubulin
	HDAC10	-
IV	HDAC11	-

Table 1.2: A summary of germ-line deletion of HDAC phenotypes in mice. (Adapted from Haberland, M., et al., 2009b)

1.8 Conditional HDAC1/2 knockout studies in mice

As discussed, deletion of both HDAC1 and HDAC2 leads to lethal phenotypes in mice, therefore, conditional deletion of *Hdac1* and *Hdac2* alleles were created using standard Cre/LoxP technology, which allows an analysis of their functions in a tissue specific manner. In addition, constitutive and conditional knockout cell lines have also been generated. HDAC1-null embryonic stem (ES) cells show reduced proliferation capacity and increased expression level of the cyclin-dependent kinase inhibitor p21, that is associated with a hyperacetylation of the histone H3 and H4 at the p21 promoter (Lagger, G., et al., 2002). Disruption of p21 in HDAC1-null mES cells rescued the reduced proliferation phenotype (Zupkowitz, G., et al., 2010). However, it was not sufficient to rescue the developmental phenotype, since *Hdac1/p21* double knockout mice are still embryonic lethal, suggesting that the developmental defects observed in the Hdac1-null mice is not due to proliferation defects (Zupkowitz, G., et al., 2010). HDAC1 also plays an essential role in the differentiation of mES cells. HDAC1-deficient mES cells exhibit precocious differentiation identified by elevated expression of cardiomyocyte and neural markers in EBs (embryoid bodies) (Dovey, O., et al., 2010). However, deletion of HDAC2 in ES cells did not yield that same phenotype, suggesting that HDAC1 is the predominant enzyme in this cell type.

Interestingly, tissue specific deletion of either HDAC-1 or -2 alone did not produce obvious phenotype, suggesting a redundant function of these two enzymes (reviewed by Kelly and Cowley, 2013). Whereas, deletion of both HDAC1/2 produced a profound phenotype in a variety of tissues, including epidermis, T-cells and B-cells (LeBoeuf, M.,et al., 2010 ; Dovey, O., et al., 2013 ; Yamaguchi, T., et al.,2010). Indeed, singular deletion of HDAC1 results in compensatory expression of HDAC2.

Dovey et al., found that, conditional deletion of both HDAC1/2 (DKO) in T-cells caused development arrest, whereas, deletion of either enzymes alone did not produce a noticeable phenotype. DKO mice exhibited a 5-fold reduction in thymocyte cellularity and down-regulation of T-cell receptor signalling components. In addition, development arrest in T-cells results in lethality at approximately 15 weeks, as of neoplastic transformation of immature T-cells (Dovey, O., et al., 2013). Another study has indicated the crucial role of HDAC1 and HDAC2 in the regulation of cell cycle. Deletion of HDAC1 and HDAC2 in mouse embryonic fibroblasts (MEFs) results in growth arrest and cell cycle block in G1-phase that is associated with up-regulation of cell cycle inhibitors p21 and p57 (Yamaguchi, T., et al., 2010). ChIP experiments also indicated the binding of both HDAC1 and HDAC2 to the promoters of P21 and P57, functional knock-down of these cell cycle inhibitors rescue the cell cycle block. Deletion of both HDAC1 and HDAC2 block B-cell development at the pre-BII stage associated with G1-phase cell cycle arrest and increased apoptosis. However, deletion of enzymes in mature resting B-cells has no negative effect on cell viability unless cells induced to proliferate they undergo rapid apoptosis (Yamaguchi, T., et al., 2010).

HDAC1 and HDAC2 are also required for development of the central nervous system (CNS). Deletion of either HDAC1 or HDAC2 alone has no deleterious effect on neuronal development. However, deletion of both HDAC1 and 2 in neurons results in hippocampal abnormalities, loss of foliation of the cerebellum, failure of differentiation of neuron precursors and lethality by postnatal day7 (Montgomery, R., et al., 2009).

1.9 Mouse embryonic stem (mES) cells

The mouse embryo 3.5 days after fertilization forms a blastocyst that is segregated into cells of the inner cell mas (ICM), which will subsequently develop into the embryo, and trophectoderm (TE), which will form the placenta. Mouse embryonic stem cells (ES cells) are derived from the inner cell mass (ICM) the day E3.5 blastocyst (Figure 1.5). In 1981, the derivation of first ES cells from mouse embryo was achieved through explanting ICM onto a feeder layer of mouse embryonic fibroblasts (Evans, M., and Kaufman, M., 1981, Martin, G., 1981). Stem cells have two distinctive properties: the ability to self-renew, they are capable of dividing indefinitely, and the capacity to differentiate into all cell types, defined as pluripotency. Importantly, ES in culture also retain the ability to differentiate into the three primary germ layers, therefore serving as a model system to recapitulate the events of early embryonic development (Doetschman, T., et al., 1985; Smith, A., 2001). ES cells can also be genetically modified which facilitates the investigation of loss of gene function in the early embryo. Moreover, the potential capacity of ES cells to produce an unlimited number of homogeneous cells indefinitely that have a normal diploid karyotype is another advantage of using these cells. Pluripotency and lineage-specific differentiation of ES cells are achieved by regulating gene activity through remodeling chromatin structure, therefore, ES cells are an excellent model to study the roles of modifying enzymes during embryonic development.

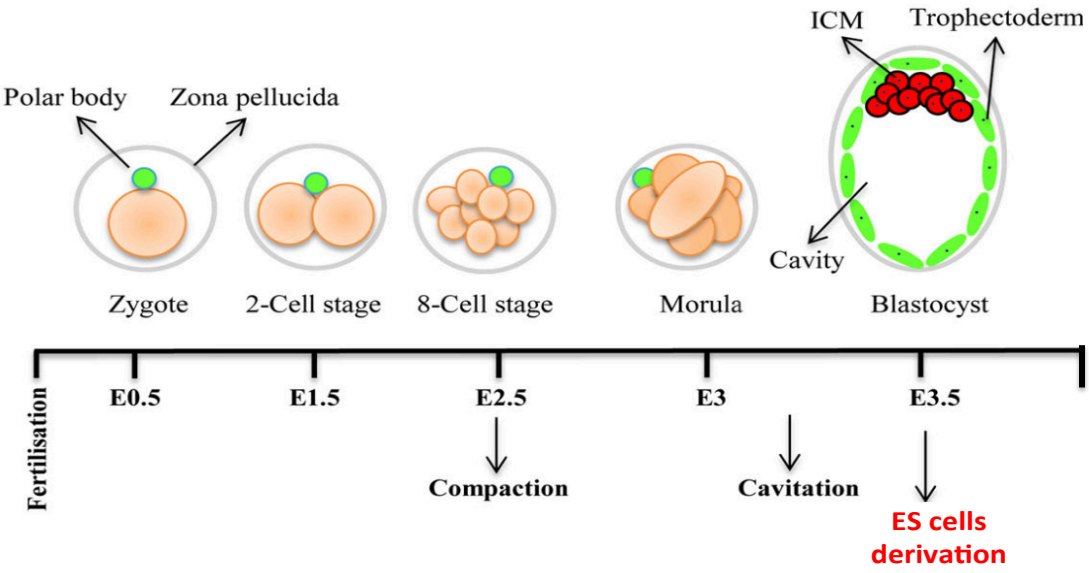


Figure 1.5: Origin of mouse ES cells derived from the ICM of the blastocyst (E 3.5). After fertilization, the zygote develops into a blastocyst through series of cleavage divisions (2-6 cell stage). ES cells are derived from the ICM cells at E3.5 (Huang, G., et al., 2015).

1.9.1 Maintenance of pluripotency

Originally, mouse embryonic stem cells were maintained in co-culture with a feeder layer of mouse embryonic fibroblasts (MEFs), supplemented with a cytokine called leukemia inhibitory factor (LIF), which promotes self-renewal of ES cells (Smith, A., et al., 1988). LIF belongs to the interleukin-6 family of cytokines that initiates signaling via its receptor gp130, resulting in activation of the STAT3 (signal transducer and activator transcription 3) pathway (Niwa, H., et al., 1998). The presence of serum or BMP4 (bone morphogenic protein4) is required to coordinate with LIF to maintain pluripotency of mES cells. BMP4 signals through SMAD activation, which

promotes expression of Inhibitor-of-differentiation (Id) proteins that suppress ectodermal differentiation (Ying, QL., et al. 2003). ES cells produce FGF4 that leads to the activation of MEK/ERK signaling pathway in an autocrine manner. The FGF/MEK/ERK signaling pathway is suggested to promote differentiation of ES cells as deletion of FGF4 restricts the differentiation ability of ES cells (Kunath, T., et al., 2007). Ying et al found that, the maintenance of self-renewal can be achieved by blocking differentiation-inducing signaling without requirement of LIF or serum/BMP4. A defined culture media was developed to maintain pluripotent state of ES cells using three small-molecule inhibitors (3i media): SU5402, inhibits FGF receptor; PD184352, inhibits MEK; and CHIR99021, inhibits GSK3 kinase. (Ying, QL., et al., 2008). GSK3 inhibitor maintains self-renewal through Wnt signaling pathway, which promotes stabilization and activation of β-catenin. Recently a combined inhibition of MEK and GSK3 (2i media) have been identified to promote naive ES cells (Leitch, H., et al., 2013). Culture ES cells under 2i condition is suggested to establish the ground state of pluripotency (more epiblast) characterized by generating homogeneous morphology and low level of DNA methylation, in which de novo methyletransferases Dnmt3a, Dnmt3b and Dnmt3l were downregulated. (Leitch, H., et al., 2013).

1.9.2 pluripotency factors

The expression of key transcription factors is essential for the maintenance of the ICM during mouse development and for maintenance of self-renewal and pluripotency of ES cells. Oct4 (Pou5f1) is a POU domain-containing transcription factor, its expression is restricted to the inner cell mass and it has been identified as a crucial factor for ICM pluripotent identity (Niwa, H., et al., 2000). Indeed, ES cells lacking OCT4 differentiate inappropriately into trophectoderm. However, overexpression of Oct4 induces differentiation toward extra-embryonic endoderm and mesoderm linages. This implicates that the restriction of Oct4 expression is required for pluripotency (Niwa, H., et al., 2000). Nanog is a homeodomain transcription factor that plays an important role in the maintenance of pluripotency in epiblast and ES cells. Loss of Nanog in ES cells results in spontaneous differentiation into primitive endoderm. Conversely, over-expression of Nanog induces ES cell self-renewal even in the absence of LIF (Mitsui, K., et al., 2003, Chambers, I., et al., 2003). Sox2 is a high mobility group (HMG)-box transcription factor that acts cooperatively with OCT4 to maintain pluripotency and self-renewal by regulating expression of multiple genes including FGF4. Unlike Oct4, expression of Sox2 is not restricted to pluripotent cells it also expressed in early primitive ectoderm (Avilion, A., et al., 2003).

A Genome-wide ChIP analysis used to map binding sites of Oct4, Nanog and Sox2 throughout human and mouse ES cells revealed that many of their target genes overlap (Boyer, L., et al., 2005; Loh, YH. , et al., 2006). In mouse ES cells, 1083 and 3006 binding sites are targeted by Oct4 and Nanog respectively, of which 345 genes are bound by both. Most of these genes encode transcription factors, including Oct4, Nanog and Sox2 themselves, as well others factors that promote pluripotency and

inhibit differentiation of ES cells. Overall, core pluripotency factors act cooperatively to construct a regulatory circuitry consisting of auto-regulatory and feed-forward loops of regulation (Boyer, L., et al., 2005; Loh, YH., et al., 2006). Core pluripotency factors are believed to be critical for repression differentiation programme. Nanog and Oct4 activate genes encoding transcription factors that mediate gene repression including, Esrrb, Rif1 and REST. Knockdown of these genes has been shown to promote ES cell differentiation (Loh, YH., et al., 2006). Moreover, core pluripotency factors regulate activation of genes encoding a component histone-modifying complex, such as Jmjd1a and Jmjd2c which are demethylase enzymes that are regulated by Oct4, depletion both of them induces ES cell differentiation (Loh., YH., et al., 2007). Perturbation of the balance between pluripotency and differentiation factors favors of differentiation and leads to lose of pluripotency. For example, a loss of balance between Nanog and Gata4 or Gata6, by decreasing Nanog or over-expression of Gata4 or Gata6, will lead to differentiation of primitive endoderm (Mitsui, K., et al., 2003; Fujikura, J., et al., 2002). Transcription factors thus work in a mutually antagonist way to regulate cell fate determination within the blastocyst, typified by the function of Cdx2 (caudal-type homeodomain transcription factor) which is required for trophectoderm, which counters the gene expression programme of Oct4 in the ICM (Niwa, H., et al., 2005).

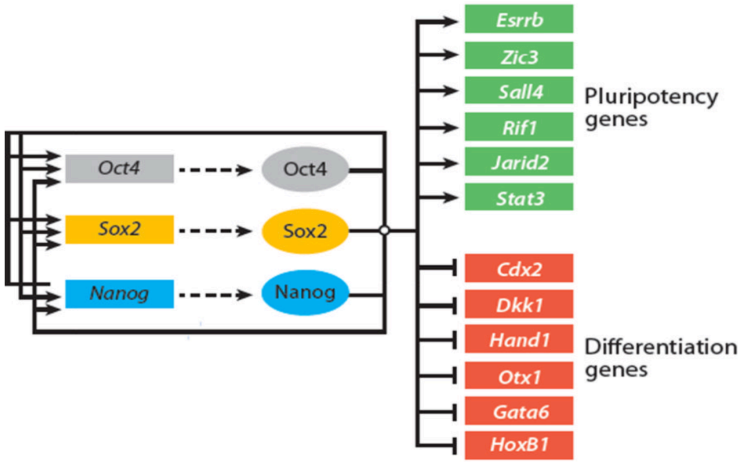


Figure1.6: Transcriptional network maintaining pluripotency in mouse ES cells. The core pluripotency factors Oct4, Nanog and Sox2 work cooperatively to regulate expression of other pluripotency factors (including themselves), while repressing differentiation genes. Figure from Loh, YH.,et al., 2011.

A recent analysis revealed an expanded set of transcription factors network containing the core pluripotent factors (Nanog, Oct4 and Sox2) in addition to other factors that control the pluripotent state of ES cells (Kim, J., et al., 2008). The nine transcription factors (Oct4, Nanog, Sox2, Dax1, Zpf281, Nac1, Klf4, Rex1 and Myc) occupy more than a third of mouse promoters in different combinations. Previously, Loh et al. found that 345 genes were co-occupied by pluripotency core factors (Nanog, Oct4 and Sox2), However, more than 800 promoters were found to be occupied by at least four of the nine transcription factors (Kim, J., et al., 2008). Moreover, the level of expression was found to correlate with number of bound factors; promoters bound by more than one factor are more likely to be transcriptionally active, whereas those bound by fewer

factors were generally repressed, including ~ 50% of genes occupied by only one factors. It also found that, Myc (occupied 18% of all promotors) and Rex1were distinct from the other factors as they highly bound active genes involved in protein metabolism. The remaining factors are enriched in genes implicated in developmental processes (Kim, J., et al., 2008).

1.9.3 Differentiation of mES cells in culture

ES cells offer the potential to study the gene expression and signaling events of early embryogenesis in a tissue culture system. ES cells have the capacity to differentiate under appropriate conditions to generate different lineages that facilitate the investigation of several aspects of early development in vitro. The first in vitro model of embryogenesis was based on differentiation of ES cells into a three embryonic germ layers: mesoderm, endoderm and ectoderm by generation of embryoid bodies (EBs), which mimic the early post-implantation embryo (Doetschman, T., et al., 1985; Keller, G., 1995).

Three general approaches are commonly used to initiate differentiation of ES cells in vitro. The first most common method involves aggregation of ES cells in suspension to form embryoid bodies (EBs) in the absence of LIF. Formation of EBs begins with the specification of the outer layer toward primitive endoderm and other lineages derived from the core of the structure (Doetschman, T., et al., 1985; Keller, G., 1995). The second method involves direct culture of ES cells on stromal cells, most often OP9 cells, in which the differentiation is initiated by cell-cell contact (Nakano, T., et al.,

1994). The third approach involves inducing differentiation of ES cells in a monolayer on extracellular matrix protein (Nishikawa, S., et al., 1998). Cells within EBs will differentiate to more advanced, committed cell types including, cardiomyocytes, neuronal cells and haematopoietic precursors. In addition, removal of LIF and serum (BMP4) will promote spontaneous differentiation of ES. Removal of LIF alleviates the inhibitory effect of STAT3 leading to differentiation towards endoderm and mesoderm, while removal of BMP4 relieves the inhibitory effect of Id protein leading to neuroectoderm differentiation. In addition, the addition of growth factors helps direct differentiation of ES cells toward specific lineages, examples include retinoic acid (RA), insulin and Wnt proteins.

1.10 Chromatin state of embryonic stem (ES) cells

ES cell Chromatin displays characteristics of transcriptionally permissive euchromatin, such an abundance of acetylated histones and increased accessibility to nucleases (Boyer, L., et al., 2006). Upon differentiation, chromatin associated with repressed genes is modified into facultative heterochromatin with a decrease in histone acetylation. Analysis of global chromatin dynamic by measuring the binding of chromatin-associated protein using fluorescent recovery after photobleach (FRAP) revealed that heterochromatin associated protein (HP1) and other histone variants are hyper-dynamically bound with chromatin of pluripotent cells and become tightly associated with chromatin in differentiated cells (Phair, R., et al., 2004; Meshorer, E.,

et al., 2006). Moreover, It has shown that, replacement of histone H1 with aversion that binds tightly to chromatin inhibited differentiation of ES cells. These data indicate that pluripotent ES cells are maintain an open arrangement of chromatin structure that becomes more compact in differentiated cells.

ES cell pluripotency is characterized by a specific epigenetic profile where lineage specific genes remain in a semi-permissive transcriptional state. These genes are typically not expressed in ES cells but become activated upon differentiation, are enriched for dual marks or bivalent domains, consisting of repressive H3K27me3 and activating H3K4me3 modifications (Azuara,V., et al., 2006, Bernstein, B., et al., 2006).The bivalent domain may promote ES cell pluripotency by maintaining the expression of lineage-specific genes in a quiescent or poised state for activation which then resolved appropriately depending on the cell type.

Most transcription factors encoding genes with a role in developmental processes have a bivalent domains bound by pluripotency factors (Oct4, Nanog, Sox2) and are also target of Polycomb repressive complex 2 (PRC2) which catalyses tri-methylation of H3K27me3. Deletion of key components of PRC2 results in de-repression of most developmental regulators genes, including HOX genes, and leads to differentiation of ES cells (Azuara,V., et al., 2006; Boyer, L., et al., 2005 ; Chamberlain, S., et al., 2008). This indicates that maintenance of a poised state is necessary for differentiation programs and indicates that histone modifying enzymes have a role in maintaining pluripotency. The histone demethylases Jmjd1a and Jmjd2c, which are positively

regulated by Oct4, are required for the expression of Nanog through demethylation of repressive marks H3K9me2 and H3K9me3. Depletion of both enzymes results in differentiation of ES cells (Loh, Y., et al., 2007). The orphan nuclear receptor GCNF (germ cell nuclear factor), which is a transcriptional repressor, mediates repression of Oct4 and Nanog through direct promoter binding, or indirectly for SOX2. Depletion of GCNF inhibits repression of Oct4 upon differentiation (Gu, P., et al., 2005). The mechanism of Oct4 repression was identified by Feldman et al., in which the targeting of the H3K9 methyletransferase, G9a to the Oct4 promoter initiates heterochromatinisation through the binding of HP1 and then recruitment of de novo DNA methyltransferases Dnmt3a/b (Feldman, N., et al., 2006).

Deletion of a component of the HDAC1/2-containing complex, NuRD, prevents repression of Oct4. ES cells lacking Mbd3 are unable to differentiate upon withdrawal of LIF (Kaji, K., et al., 2006). Sin3A-HDAC corepressor complex was found to positively regulate expression of Nanog in ES cells. Knockdown of mSin3A leads to reduction in the expression level of Nanog (Baltus, G., et al., 2009). HDAC1 was found to effect differentiation of ES cells. Deletion of HDAC1 in ES cells results in precocious differentiation that is identified by elevated expression of cardiomyocyte and neural markers in EBs (embryoid bodies) (Dovey, O., et al., 2010). Moreover, another study found that treatment of day 7 EBs with TSA (HDAC inhibitor) promotes differentiation into cardiomyocytes that is identified by induced expression of Nkx2.5 (Kawamura, T., et al., 2005). Collectively these data indicate a functional role of HDAC-containing complexes in embryonic gene regulation.

1.11 Aims of the project

Many mouse knockout studies have demonstrated the essential role of HDAC1/2 in the development of numerous tissues (discussed in section 1.8). Moreover, HDAC1 and HDAC2 are functionally redundant in most cell types, deletion of both HDAC1/2 is required to produce a profound phenotype. In this project two model systems were used to investigate the role of HDAC1 and HDAC2 in ES cells. Firstly, a compound deletion of *Hdac1* and *Hdac2* (*Hdac1* $^{Lox/Lox}$*; Hdac2* $^{Lox/WT}$*; CreER* ES cell line (in which only a single copy of HDAC2 remains) is used a model system in which HDAC1/2 activity is decreased but not entirely lost. Secondly, a double conditional knockout (DKO) *Hdac*$1^{Lox/Lox}$; *Hdac*$2^{Lox/Lox}$; CreER ES cell line is used to circumvent the functional redundancy between HDAC1/2.

Therefore, using these two model systems the aims of the project were:

- To investigate the effect of deletion during proliferation and differentiation of ES cells.
- To assess the biochemical properties of ES cells lacking HDAC1/2 and their contribution to the regulation of the ES cell transcriptome.
- To investigate the positive role of HDAC1/2 in regulating gene expression.

Chapter 2: Materials and Methods

2.1 Generation of *Hdac1*, *Hdac2* double knockout (DKO) ES cells.

The conditional *Hdac1, Hdac 2* knockout ES cells used in this thesis were generated by Dr. Shaun Cowley. E14 ES cells expressing a CreER fusion protein from the ROSA26 locus were used to generate *Hdac*1$^{Lox/Lox}$; *Hdac2*$^{Lox/Wt}$; CreER ES cells and *Hdac*1$^{Lox/Lox}$; *Hdac2*$^{Lox/Lox}$; CreER conditional knockout ES cells, using multiple rounds of gene targeting (Jamaladdin, S. et al, 2014). LoxP sites were placed flanking exon 2 of each gene. Addition of 4-hydroxytamoxifen (4-OHT) to the growth media induced Cre-recombinase activity and resulted in deletion of exon 2. The deletion of exon 2 disrupts the open reading frame of HDAC1 and HDAC2 and a premature stop codon is introduced into exon 3.

2.2 Culture and maintenance of mouse ES cells

2.2.1 Thawing and plating of mES cells

Cryovials of mES cells were removed from liquid nitrogen and thawed rapidly in a 37°C waterbath. The 1ml of thawed cells was transferred to a 15ml falcon tube and 4ml of ES cell media were added. The cells were then pelleted by centrifugation for 5mins at 1200rpm. The cell pellet was re-suspended in M15 ES cell media and plated onto 10cm^2 tissue culture plate coated with 0.1% gelatin solution in PBS. ES cells were subsequently maintained in standard ES cell medium (M15+LIF) and were grown in a 5% CO_2 incubator at 37°C.

2.2.2 Passage of mES cells

ES cells were routinely passaged every two days. Culture media was aspirated and cells were washed twice with room temperature PBS. An appropriate amount of trypsin solution was added (3ml for 10cm^2 plate), cells were incubated for 5mins at 37°C. To inactivate the trypsin, 6ml of standard mES cell medium (M15+LIF) were added and cells were suspended by pipetting up and down several times to separate the cells. Cells were centrifuged for 5mins at 1200rpm, and cells pellet was re-suspended in fresh media and split into gelatinized plates. The plated cells were mix carefully (sideways) to distribute the cells evenly.

2.2.3 Freezing of mES cells.

ES cells were frozen from an 80% confluent 10cm^2 plate that yields approximately 3 x 10^7 cells. ES cells were trypsinised as described in (2.2.2) and re-suspended in equal volumes of 2x freezing media and ES cell media. 1ml aliquot of cells were transferred to 1.5ml freezing cryovials and placed into a freezing pot containing iso-propanol and placed at -80C (cells will freeze at 1°C per minute). After 1-2 days, cryovials were transferred to liquid nitrogen for long-term storage.

2.3 Media and reagents used for culture of ES cells

M15+LIF ES cell medium

Knockout DMEM (GIBCO, Life Technologies)	500ml
Foetal Bovine Serum (Seralab)	90ml
100X Glutamine/Pencillin/Streptomycin (Gibco)	6ml
100mM β-mercaptoethanol	600μL
Leukaemia Inhibitory Factor (LIF, Synthesized In House)	40 μL

0.1% Gelatin

PBS (GIBCO)	500ml
2% Bovine gelatin solution	25ml

2X Freezing media

Knockout DMEM (GIBCO, Life Technologies)	60%
Foetal Bovine Serum (Seralab)	20%
DMSO (Invitrogen)	20%

EB media (M15)

Knockout DMEM (GIBCO, Life Technologies)	500ml
Foetal Bovine Serum (Seralab)	90ml
100X Glutamine/Pencillin/Streptomycin	6ml
100mM β-mercaptoethanol	600µL

Retinoic acid differentiation media (RA)

M15+ LIF	500ml
100mM all trans-Retinoic Acid (Sigma)	50 µL

N2B27 differentiation media (50ml)

Knockout DMEM/F12 (GIBCO)	50ml
N-2 supplement (Invitrogen)	500 µL
B-27 supplement (Invitrogen)	1000 µL
Recombinant mouse FGF basic (R and D system)	10 µL (50µg/ml stock)

2.4 Protein and enzymatic analysis

2.4.1 Protein extraction

ES cells were cultured until 80% confluent in 10cm^2 plates, media was removed and plates were washed twice with 1x PBS and then scraped in 1ml PBS. Samples were pelleted at 1200rpm, re-suspended in 400µl ice-cold IP buffer and placed in rotator for 20mins at 4°C. Samples were spun for 20mins at 14,000 rpm in a 4°C pre-cooled centrifuge and the supernatant transferred to a fresh 1.5ml tube. Protein concentration was quantified using Bradford reagent (BIO-RAD) and a standard spectrophotometer.

IP Buffer

250mM NaCL

20mM HEPES (pH 7.4)

0.5%(v/v) IGEPAL

1X Protease inhibitor cocktail (Sigma)

2.4.2 Western blotting

Protein samples were prepared for electrophoresis by using 35µg of protein with an equal volume of 2x protein loading buffer. Samples were boiled at 100°C for 5mints to denature the protein and loaded into the 4-12% SDS-PAGE gel and run for approximately 1hour at 150 V. The gel was placed in the transfer sandwich (foam pad-filter paper-gel-nitrocellulose membrane-filter paper-foam pad), placed in a transfer tank filled with transfer buffer and transferred for 1 hour at 90 V.

Following transfer, the membrane was blocked with odyssey blocking buffer (LI-COR) for 1 hour at room temperature and then incubated for 1hour with appropriately diluted primary antibody in 3ml of odyssey blocking buffer (LI-COR). The membrane was washed 3 times with PBST (PBS+0.1%Tween) for 10min, followed by incubation with the appropriate IRDye conjugated secondary antibodies for 45mins. After incubation, the membrane was washed 3 times with PBST and once with PBS. Proteins were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences).

1X Running buffer

192mM Glycine

25mM Tris-base

0.1% SDS

1X Transfer buffer

192mM Glycine

25mM Tris

10% Methanol

Protein loading buffer (PLB)

70mM Tris-HCL (PH6.8)

200mM β-mercaptoehanol

2% SDS

20% Glysrol

Bromophenol Blue

2.4.3 Co-immunoprecipitation

Co-immunoprecipitation assays were performed using protein-G agarose beads (GE Life Sciences, Buckinghamshire). 50µl of beads were washed twice with ice-cold PBS and incubated with 1µg of antibody for 20mins at 4°C. The bead-antibody mix was washed 3 times with ice-cold PBS and incubated with 600µg of protein extract overnight at 4°C. The following day, the bead-Protein complexes were washed 3 times with IP buffer and split into two aliquots, one aliquot was used to assess the enzymatic activity of the immunoprecipitates using a commercially available HDAC Assay kit (Active Motif) and the second aliquot was resolved by SDS-PAGE and probed with antibodies raised against known components of the immunoprecipitated complexes.

2.4.4 Histone extraction and analysis of post-translation modifications

Cells were harvested by scraping in 1ml PBS and whole cell extract (WCE) was isolated as described in (2.4.1). Pellets were re-suspended in 400µl 0.2M H_2SO_4 and incubated overnight with rotation at 4°C. The following day, samples were spun for 20mins at 14,000 in 4°C pre-cooled centrifuge, with the supernatant transferred to a fresh 1.5ml tube. 20µg of each histone extract was resolved by SDS-PAGE and probed with antibodies raised against a numbers of the specific histone modifications indicated. Membranes were scanned using the Odyssey Infrared Imaging System and quantification of proteins performed using the appropriate IRDye conjugated secondary antibodies (Li-COR Biosciences) .

2.4.5 Histone deacetylase assay

The HDAC assay was performed using a commercially available colorimetric kit (Active Motif), which utilizes a BoC-Lys(Ac)-AMC substrate that contains an acetylated lysine residue. Once the substrate is deacetylated, the lysine residue then reacts with the Developing Solution and releases the chromaphore from the substrate resulting in a yellow colored product that absorbs maximally at 405nm. The 80µl of immunoprecipitated protein extract generated in (2.4.3) was split into triplicates and added into 96-well plate. 20µl of HDAC assay buffer and 5µl of the colorimetric substrate were added to each well. The plate was incubated for 3 hours at 37°C. The reaction was stopped by the addition of 50µl of the developing solution, incubated for 15mins at room temperature and read using a plate reader at 405nm.

2.5 Induction of HDAC1, HDAC2 protein deletion

ES cells were plated in 10cm^2 plate with M15+LIF medium and treated with 1µM 4-hydroxytamoxifen (OHT) for 24 hours. The following day, media was changed and cells cultured for a further 4 -8 days. Protein extracts were isolated as described in (2.4.1), antibodies used in western blotting are indicated in (Appendix Table1).

2.6 ES cells growth curves

ES cells were plated at 3 x10^5 cells per well in triplicate in a 6-well plate with M15+LIF medium. Viable cells were counted over 4 days after the deletion of HDAC1, HDAC2 had been induced. Cells were counted using automated cell counter Bio-Rad TC-10.

2.7 Flow cytometry

2.7.1 Propidium iodide (PI) staining

The culture media was collected from each sample, cells were then harvested by trypsinisation and pooled with the media in 15ml tubes. Samples were centrifuged for 5mins at 1100rpm, pellets were fixed by drop-wise addition of 1ml ice-cold 70% ethanol and then either stained or stored at 4°C. Cells were subsequently washed with PBS and re-suspended in 500µl PI buffer (50µg/ml of Propidium Iodide, 10µg/ml RNaseA in 1x PBS) and incubated for 30min in the dark. FACS analysis was performed on the BD FACSCanto II and FACSDiva 6.0 software for acquisition and analysis.

2.7.2 Analysis of apoptosis using Annexin-V

Cells were harvested by trypsinization and collected in 15ml tubes, centrifuged for 5mins at 1100rpm, and then washed once with PBS. Cell pellets were re-suspended in 500µl 1x annexin-V binding buffer, 5µl of annexin-V was added (Invitrogen) and incubated for 15mins at room temperature. FACS analysis was performed on the BD FACSCanto II and FACSDiva 6.0 software for acquisition and analysis.

2.7.3 Analysis of GFP expression

Cells were harvested by trypsinisation and washed twice with PBS. Cells were pelleted at 1100rpm and re-suspended in 1ml PBS. Live cells were analyzed using BD FACSAria II and gated based on FSC and SSC, the GFP expression in each cell measured by the FITC-A channel. GFP-expressed cells were sorted and collected in 1.5ml ES cells media.

2.8 RNA isolation and q-RT-PCR

2.8.1 RNA isolation from ES cells and Embryoid bodies (EBs)

All Chemicals and equipment used in RNA isolation were treated with RNAseZap spray (Ambiob) to completely remove RNAase contamination. ES cells were harvested from 6cm plates and EBs were collected in 1.5ml tubes. For each experiment, samples were collected in TRIzol reagent (Life technologies) and stored at -80°C until RNA isolation. To isolate RNA from ES cells, plates were washed twice with PBS, 1ml of TRIzol was added directly to the plate to lyse cells and extract RNA. Samples were collected by pipetting up and down several times and transferred to 1.5ml tubes. For EBs, 500-1000µl of TRIzol was added depending on the size and number of the EBs.

Direct-zol™ RNA MiniPrep kit (ZYMO RESEARCH) was used to isolate the RNA from samples stored in TRIzol. Following thawing of -80°C stored samples, 1ml of 100% ethanol was added and mixed by vortex. Samples were loaded into a Zymo-Spin II Column in a collection tube and centrifuged for 1min. 400µl RNA wash buffer was added and centrifuged for 30s, the flow-through was discarded. Each Sample was treated with DNase I reaction mix: 5µl Dnase I, 8µl 10x DNase I reaction buffer, 3µl DNase/RNase free water, 64µl RNA wash buffer, incubated for 15min at room temperature and centrifuged for 30s. Samples were washed twice with 400µl of Direct-zol RNA Prewash and centrifuged 30s. 700µl of RNA wash buffer was added and centrifuged for 30s. The column was transferred to a new collection tube and centrifuged for 2min to ensure complete removal of wash buffer. To elute RNA, 25µl of DNase/RNase free water was added to column and centrifuged for 1min.

RNA concentration was quantified using NanoPhotometer®(Implen). Then RNA samples used in the microarray analysis underwent a second purification step using an RNeasy MinElute Cleanup Kit (Qiagen).

2.8.2 Reverse transcription

Total RNA was quantified using a NanoPhotometer® (Implen). cDNA was prepared using 0.5µg of total RNA with the Q-Script cDNA Supermix (Quanta Biosciences). To each sample the following were added, 4µl of qScript cDNA Supermix , 0.5µg of RNA and DNase/RNase free water up to a volume of 20µl. cDNA synthesis was carried out in the thermo cycler with the following temperatures:

5 minutes	25°C
30 minutes	42°C
5 minutes	85°C
Hold	4°C

The resulting cDNA was diluted with an equal volume of DEPC treated H_2O before use for RT-PCR experiments.

2.8.3 Quantitative real time PCR (qRT-PCR)

The primers used for each gene were designed using the Universal ProbeLibrary Assay Design Centre (www.roch-applied-science.com). In the multiplex PCR, GAPDH was used as a reference control to normalize the target gene Ct value. Probes consisted of Lock Nucleic Acid technology, which upon binding of the reaction amplicon and polymerase elongation released a HEX or FAM fluorophore. The multiplex reaction mix was made using the LightCycler Probes Master (Roche) as per the manufacturer's instructions. Reactions were performed in wells of white LightCycler 480 Multiwell plate 96(Roche) using 2µl of diluted cDNA per reaction. Reaction was carried out on Roche Light Cycler 480 under the following conditions:

10 minutes	94°C	
10 seconds	94°C	40 cycles
20 seconds	55°C	
5 seconds	72°C	
Hold	4°C	

Advanced relative quantification analysis using the Roche LightCycler software generated a relative expression value based on the comparative Ct calculations ([delta][delta] Ct = [delta] $Ct_{,sample}$ - [delta] $Ct_{,reference}$).

2.9 Microarray Hybridization

RNA was isolated form ES cells using a Direct-zol™ RNA MiniPrep kit (ZYMO RESEARCH) and an RNeasy MinElute Cleanup Kit (Qiagen) as described (2.8.1). Quality control of total mRNA was performed using a 2100 Bioanalyser (Agilent). Only samples that had an RNA integrity number of 8.6 or higher were selected for processing and array hybridization.

2.9.1 RNA amplification

RNA amplification was performed using an IIIumina ® TotalPrep RNA amplification kit according to manufacture's instruction which generated biotinylated, amplified RNA for Hybridization with the Illumina bead array. The kit is based on the RNA amplification protocol developed in the laboratory of James Eberwine (Vangelder *et al*., 1990). The procedure consists of reverse transcription with an oligo (dT) primer bearing a T7 promoter using a reverse transcriptase enzyme engineered to produce higher yields of first strand cDNA the wild type enzymes. This enzyme catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second strand synthesis and cleanup to become a template for in vitro transcription (IVT) with T7 RNA polymerase.

2.9.2 Array hybridization

Comparative microarray gene expression profiles were generated using the Illumina mouseWG-6, version 2.0 Beadchip that covers 45,200 different mouse transcripts. The Direct Hybridizaion Assay system uses gene-specific probes to detect labeled RNA, each bead in the array contains a 50bp gene-specific oligo probe .The labeled RNA was hybridized to the probes on the beadchip for 14-20 hours at 58°C, the beadchip was then washed twice and the signal detected using Illumina iScan system.

2.9.3 Analysis of microarray hybridization

Raw expression data was analyzed using Illumina BeadStudio software. The detection P values of <0.01 were used to filter all data. Significant differentially expressed genes were defined using fold change of ≥1.4 (Fc ≥ 1.4) with an adjusted P value of <0.05. Quality analysis and differential expression analyses were performed using Partek Genomics Suite (version 6.5) and ArrayTrack. Analysis of functionally related gene groups among deregulated genes was carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID), version 6.7.

2.10 Analysis of ES cell Pluripotency and Differentiation

2.10.1 Alkaline phosphatase assay

Initially, ES cells were plated at $5x10^2$ cells per well in 6-well plates in the presence of LIF. On the following day, cells were then cultured either in the presence or absence of LIF for 6 days to allow colonies to form. Colonies were fixed with 4% paraformaldehyde in PBS for 2mins, washed twice in PBS+ 0.1% Tween and then stained with the a commercial Alkaline Phosphatase detection Kit (Millipore): Fast Red Violet, Naphthol and water in a 2:1:1 ratio, incubated for 15mins in dark at room temperature and washed in PBS+ 0.1% Tween. Cells were visualized by light microscopy and scored undifferentiated (dark purple staining), mixed (intermediate purple staining) and differentiated (colorless).

2.10.2 Differentiation of ES cells as Embryoid Bodies (EBs)

Embryoid bodies (EBs) were created by plating $7x10^2$ cells per well in Corning® Costar® Ultra-Low attachment round bottom 96 wells plate (Sigma-Aldrish). EBs were cultured in M15 medium (-LIF) for 12 days. EBs were visualized every 2 days by light microscopy and diameters measured using the Leica Application Suite software.

2.10.3 Differentiation of ES cells with Retinoic Acid (RA)

To induce differentiation and cell cycle withdrawal, *Hdac1/2* of DKO ES cells (1.5 x10^5 cells) were plated in triplicate in 6cm^2 plates and treated for two days with 1μM retinoic acid (RA) diluted in M15 media. After two days cultures in the presence of RA, cells were counted each day over 4 days using an automated cell counter (Bio-Rad TC-10).

For microarray experiments, 3X10^5 ES cells were plated in 6cm^2 plates and treated with 1μM 4-hydroxytamoxifen (OHT) for 24 hours, 2.5 days later, 1μM retinoic acid (RA) was added for 6hrs. 1ml of TRIzol was added directly to the plate, pipetting the cells up and down several times to lyse the cells, before transferring to 1.5ml tubes and storage at -80°C until the RNA was isolated as described in (2.8.1)

2.10.4 Differentiation of ES cells in LIF-free medium

7x10^5 ES cells were plated in 6cm^2 plate in M15 media (-LIF) for 4 days. 1ml of TRIzol was added and RNA was isolated as described in (2.8.1).

2.10.5 Differentiation of ES cells in serum-free N2B27 media

ES cells were plated with N2B27 media in 6cm^2 plate coated with laminin. Plates were coated with 2ml(10µg/ml) Natural Mouse Laminin (Invitrogen) and incubated at 37°C for 3 hours. Differential cell numbers were plated: day 0, 1x10$^{6;}$ day 2, 5x10$^{5;}$ day4, 2x10^5; day6, 1.3x10^5. Cells were collected in TRIzol and RNA was isolated as described in (2.8.1).

2.11 Plasmid Transfection

2.11.1 Transformation and culture of bacterial cells

Different histone deacetylase 1 (HDAC1) mutants and chimeric cDNAs were generated by PCR and sub-cloned into a pCAG-IRES-eGFP plasmid using In-Fusion HD EcoDry Cloning Plus kit (Clontech). For transformation, 50µl of α-select competent cells (Bioline) were thawed on ice and mixed with 1µl of plasmid. Cells were incubated on ice for 30min and heat shocked for 30s at 42°C then placed on ice for 2mins. 950µl of SOC medium were added. Bacterial cells were grown at 37°C for 1 hour in a shaking incubator, then 5µl of transformed cells spread on LB agar plate containing Ampicillin and incubated overnight at 37°C. For Maxipreps, bacterial colonies were picked from agar plates and used to inoculate of 100ml of LB media containing the appropriate antibiotic and incubated overnight in 37°C shaker. The EndoFree Plasmid Maxi Kit (Qiagen) was used for plasmid isolation as per manufacturer's instructions.

2.11.2 Plasmid transfection

Transfection of ES cells with plasmids was performed using Lipofectamine 2000 (Invitrogen). A day before transfection, $2.5x10^5$ cells were plated in 6-well plates. For each transfection, 12µl of lipofectamine was mixed with 250µl of DMEM in an Eppendorf tube and incubated at room temperature. In another tube, 5µg of DNA was added to 250µl of DMEM. After 5 mins incubation, the diluted DNA and diluted lipofectamine were combined together, mixed and incubated for 20mins at room temperature. After incubation, 500µl of the mixture was pipetted drop-wise into the culture medium.

2.12 Rescue of KO cells

Rescue of *Hdac1/2* DKO ES cells was performed using different cDNA plasmids. DKO ES cells were transfected with 5μg of DNA plasmids using Lipofectamine 2000 (Invitrogen) as described in (2.11.2). Cells were cultured for 48 hours in 5% CO_2 incubator at 37°C before sorting for GFP-positive cells using BD FACSAria II as described in (2.7.3). GFP-positive cells were plated in triplicate in 96-well plate with M15+LIF medium and treated with 1μM 4-hydroxytamoxifen (OHT) for 24 hours. Cells were cultured for 4 days; viable cells were counted using automated cell counter Bio-Rad TC-10.

Chapter 3: Examination of proliferation and differentiation potential of *Hdac1* $^{Lox/Lox}$; *Hdac2* $^{Lox/WT}$; *CreER* Embryonic stem cells

3.1 Chapter aims

The class I HDACs, HDAC1 and HDAC2 are highly similar enzymes (82% identical), which are present in the multiprotein co-repressor complexes Sin3a, NuRD, and CoREST. Disruption of *Hdac1* gene in mice results in embryonic lethality around embryonic day E10.5 due to severe proliferation defects (Lagger, G., et al., 2002). In another study, it has shown that deletion of HDAC1 results in embryonic lethality by E9.5. In contrast, mice lacking HDAC2 survive embryogenesis and died 24 hours after birth due to cardiac defects (Montgomery, R., et al., 2007), or survive to adulthood in two further studies (Trivedi, C.M. et al., 2007 and Zimmermann, S., *et al.,* 2007). Moreover, conditional deletion of *Hdac1* in mouse ES cells causes enhanced differentiation of embryoid bodies EBs compared to control and *Hdac2*- deficient ES cells, the differentiation characterized by increased expression of cardiomyocyte and neural markers (Dovey, O.M. et al, 2010). These results suggest the essential role of HDAC1 at earlier developmental stage. In many cell types, deletion of both *Hdac1* and *Hdac2* is required to produce a profound phenotype. For instance, cardiac deletion of *Hdac1* or *Hdac2* has no effect while deletion of both *Hdac1/2* causes dilated cardiomyopathy and neonatal lethality (Montgomery, R., et al., 2007). These results suggest a functional redundancy between the function of HDAC1 and HDAC2 and degree of compensation in their expression.

Therefore, in this chapter I aimed to investigate a compound deletion of *Hdac1* and *Hdac2* (*Hdac1*^{ko}*; Hdac2*^{Het}) during the differentiation of ES cells, as model system of the events of the early embryogenesis.

3.2 Results

3.2.1 Generation of conditional knockout ES cells

An E14 ES cell line expressing a Cre/estrogen receptor (CreER) fusion from ROSA26 locus was used to generate *Hdac1*$^{Lox/Lox}$*; Hdac2*$^{Lox/WT}$*; CreER* ES cell line using multiple rounds of gene targeting (Figure 3.1A). The Cre-Lox system relies on site-specific recombination; Cre (causes recombination) is a 38kDa protein from bacteriophage p1 that catalyzes recombination between pairs of LoxP (locus of X over P1) sites. A LoxP site is 34bp in size and consists of two 13bp inverted repeats separated by 8bp asymmetric region .The recombination depends on the orientation of loxP sites. Inverted *loxP* sites will cause an inversion, while a direct repeat will cause a deletion of the DNA sequence between pairs of LoxP sites.

To achieve the conditional knockout gene targeting, Cre fused to a mutated ligand-binding domain (LBD) of the estrogen receptor (ER), in which Glycine 521 mutated to Arginine. The mutated CreER is only activated by 4-hydroxytamoxifen (4-OHT) and is unresponsive to endogenous 17 *β-estradiol* (E2) (Sauer, et al. 1988).

Using homologous recombination, we have generated *Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/WT}$; CreER ES cell line, in which exon 2 of *Hdac1* (both alleles) and *Hdac2* (one allele) is flanked by LoxP sites (Figure 3.1). Addition of 4-hydroxytamoxifen (OHT) for 24hours to the growth media induced translocation of the Cre/ER into the nucleus and mediated recombination of *loxP* sites that resulted in deletion of exon 2 (Figure 3.1). The deletion of exon 2 disrupts the open reading frame of HDAC1 and HDAC2 and a premature stop codon is introduced into exon3, which is subjected to nonsense-

mediated decay or produces a non-functional protein that lacks the catalytic deacetylase domain in both HDAC1 and HDAC2.

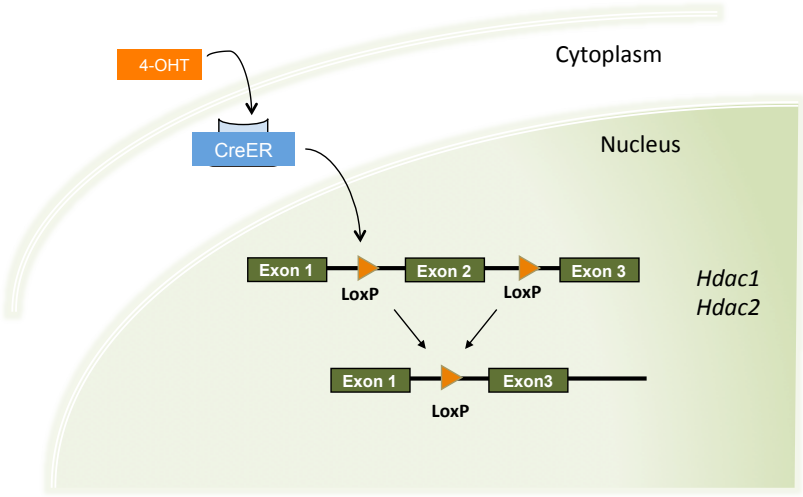


Figure 3.1: Schematic of conditional knockout system. Activation of CreER by adding 4-OHT that results in translocation of Cre to the nucleus which catalyzes recombination between two LoxP sites and results in deletion of exon 2 of *Hdac1* and *Hdac2* genes.

Following inactivation of *Hdac1 (KO)* and *Hdac2 (Het)* genes protein levels of HDAC1 and HDAC2 were analyzed by quantitative western blotting of control (untreated) and KO (OHT-treated) cells over an 8 day time-course. As seen in figure 3.2, following OHT treatment a further 2-3 days are required for the complete loss of HDAC1 protein. We also observed a slight reduction in HDAC2 protein level, which is

in contrast *Hdac1*$^{Lox/Lox}$*; CreER* cells which show increased HDAC2 levels (Dovey, O.M. et al, 2010), which is presumably due to having only a single copy of the *Hdac2* allele (Figure 3.2B).

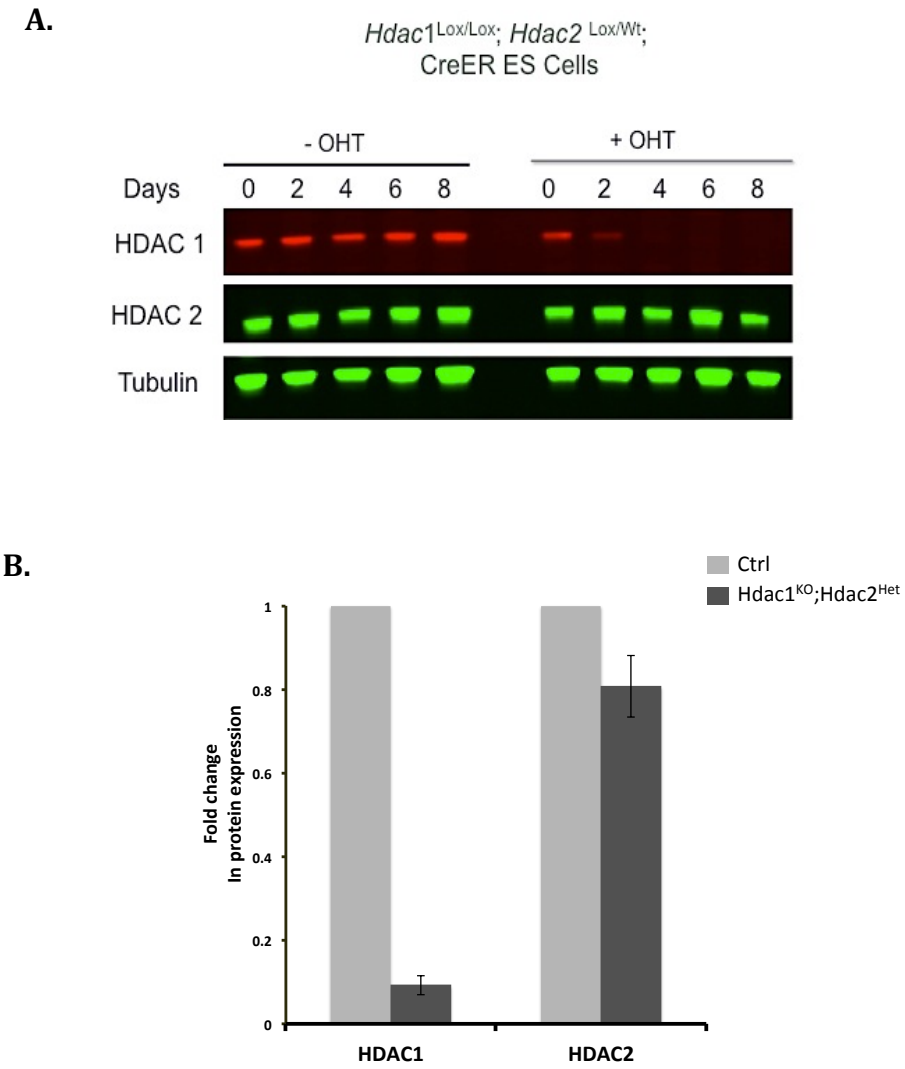


Figure 3.2: Quantification of HDAC1 and HDAC2 levels following gene inactivation. (A) Quantitative western blot showing loss of HDAC1 proteins following gene inactivation (0-8d) in *Hdac1* $^{Lox/Lox}$*; Hdac2* $^{Lox/WT}$*; CreER* ES cells. Cells were cultured with, or without, 4-OHT for 24 h. α-Tubulin was used to normalized protein loading (B) Fold change in HDAC1 and HDAC2 protein level 3d following gene inactivation relative to α-Tubulin. Western blot was visualized and quantified using odyssey scanner. All values are means (n = 3) ±SEM.

3.2.2 Decrease in total cellular deacetylase activity

A reduction in the level of HDAC1 and HDAC2 in *Hdac1*^{KO}*; Hdac2*^{Het} cells (Figure 3.2) suggested that there may be an overall reduction in total deacetylase levels. Therefore, total deacetylase activity of the cells was measured four days after genes inactivation (OHT treatment) at the point when protein levels have reached a minimum. We observed a reduction in the deacetylase activity by day 3 of approximately 56% consistent with the loss of HDAC1 protein (Figure 3.3). This result suggests that HDAC1 (in the absence of a compensatory increase in HDAC2) contributes the majority of the deacetylase activity in embryonic stem cells.

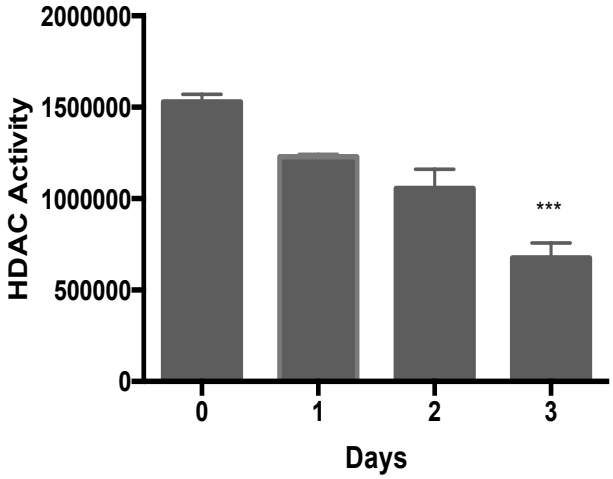
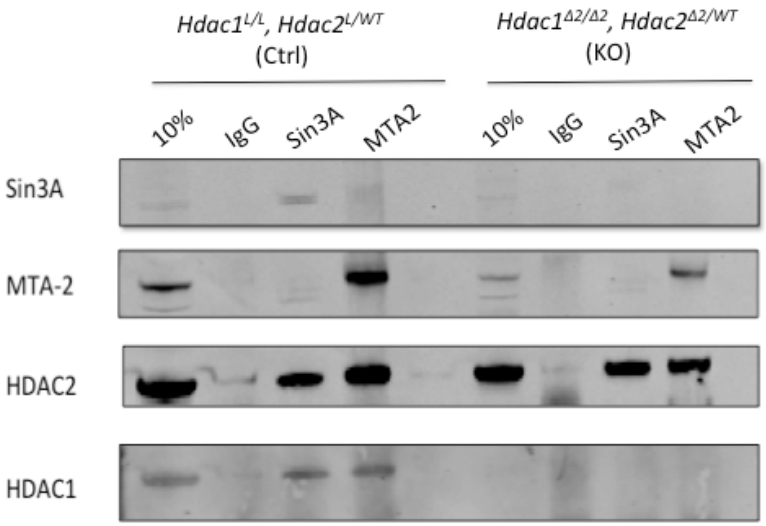


Figure 3.3: Decrease in the overall deacetylase activity in *Hdac1*$^{Lox/Lox}$; *Hdac2*$^{Lox/WT}$; *CreER* ES cells. Deacetylase activity was measured in whole-cell extract on 4 consecutive days following gene inactivation using a commercially available kit. All values are means (n = 3) ±SEM. The significant (P value) was calculated using a two-tailed t test (***$P <$ 0.0001).

3.2.3 Reduction in levels of co-repressor complex components

HDAC1 and HDAC2 are normally associated with the multi-protein complexes Sin3A, NuRD, and CoREST. To assess the integrity of the HDAC1/2 co-repressor complexes in *Hdac1*^{KO}*; Hdac2*^{Het} cells, Sin3A and MTA2 were co-immunoprecipitated with HDAC1 and HDAC2 followed by western blotting. In the KO cells the level of Sin3A and MTA2 are significantly decreased compared to the control (Figure 3.4A). To further confirm this result and test if we obtain the same reduction in the level of CoREST, western blots were also performed on protein extracts from KO cells (day3) and control (untreated). Consistent with the CO-immunoprecipitation result, the level of MTA2 is reduced as were CoREST protein levels (Figure 3.4 B). Since we observed reduction in the level of direct HDAC1/2 binding partners, it suggests that the integrity of the complexes is disrupted in *Hdac1*^{KO}*; Hdac2*^{Het} cells as a result of lacking HDAC1, despite the fact that we still detect HDAC2 (50% reduction compare to WT) (Figure 3.2).

A.



B.

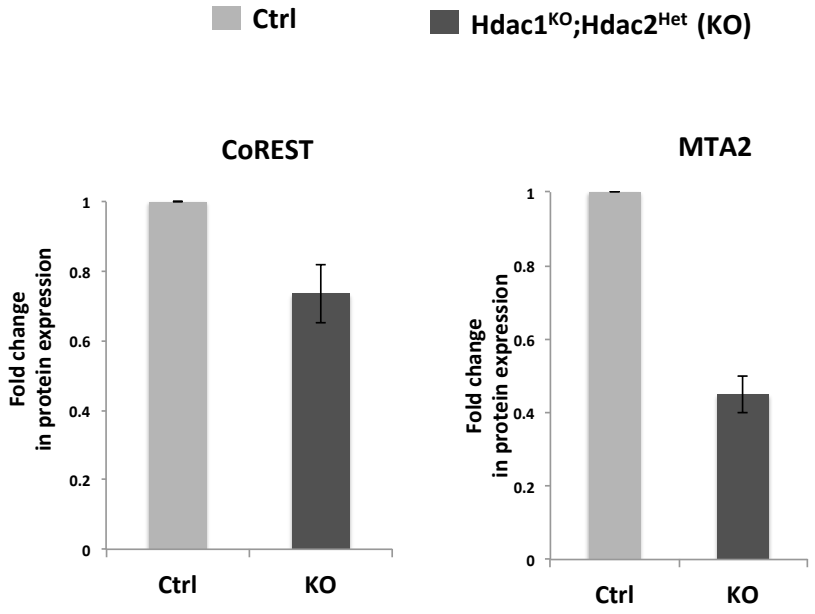


Figure 3.4: Reduction in Sin3A, MTA2, and CoREST protein levels in *Hdac1* $^{Lox/Lox}$; *Hdac2* $^{Lox/WT}$; CreER ES cells. (A) Specific antibodies to the indicated proteins were used to immunoprecipitate Sin3A and MTA2 from untreated (Ctrl) and OHT-treated (KO) ES cells. IgG was used as a non-specific antibody control. (B) Quantitative Western blotting for CoREST and MTA2 protein levels was performed on untreated (Ctrl) and OHT-treated (KO) cells 3d following gene inactivation. Blots were quantified using an Odyssey scanner.

The reduction in the co-repressor complexes stability and reduction in total deacetylase activity prompted us to analyze global histone acetylation levels in *Hdac1*^{KO}*; Hdac2*^{Het} (KO) cells. We detected a slight change in the acetylation status of H3K18Ac, H3K9Ac, H3K23Ac and H3K27Ac (figure 3.5). In addition, we observed that H3K14Ac and H3K56Ac were increased 1.3 fold and 2.3 fold, respectively. The significant increased in the acetylation level of H3K56Ac is in agreement with previous results in *Hdac1* deleted cells (Dovey, O.M. et al, 2010). This modification is associated with DNA damage, nucleosome assembly and the activity of stem cell factors (Das C., et al., 2009, Tjeertes J.V., et al., 2009 and Xie W.,et al., 2009).

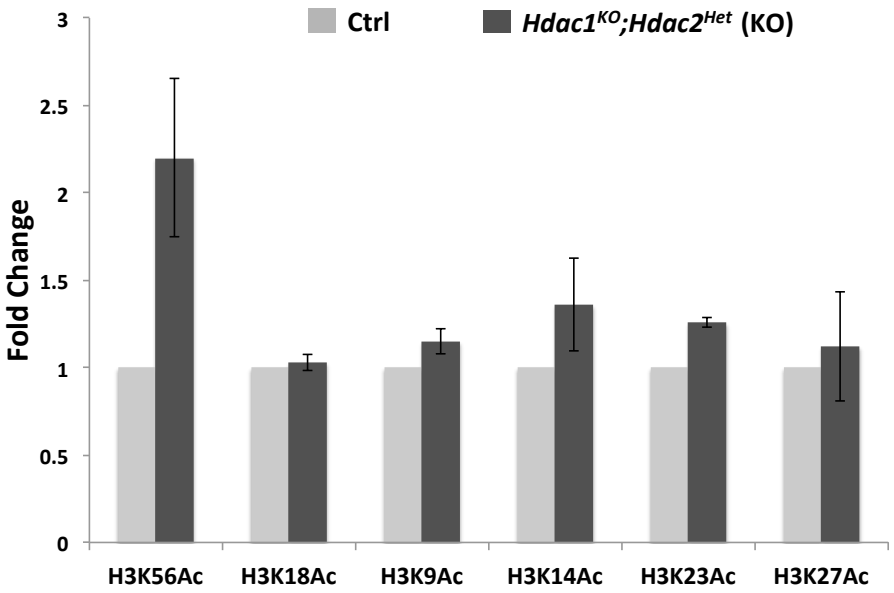


Figure 3.5: Increase in the global histone H3 acetylation levels. Quantitative Western blotting was used to determine the levels of global H3 acetylation. Histones were acid extracted from untreated (Ctrl) and OHT-treated (KO) cells 3d following gene inactivation. Acetylation levels were normalized to the total amount of H3 quantified using an Odyssey scanner. All values are means (n = 3) ±SEM.

3.2.4 Proliferation and differentiation ability of *Hdac1^{KO}; Hdac2^{Het}* cells is not inhibited

It has previously shown that the proliferation ability of ES cells is not inhibited by loss of HDAC1, or HDAC2 alone (Dovey, O.M. et al, 2010). HDAC1 has been implicated in cell cycle regulation, it is required for transcription repression mediated by retinoblastoma tumor suppressor protein, Rb (Brehm, A., et al 1998), and controls expression of specific CDK inhibitors (Lagger, G., et al., 2002 and Senese, S., et al., 2007). Therefore, the proliferative ability of *Hdac1^{KO}; Hdac2^{Het}* cells was assessed compared to controls (untreated) over a four-day period. As shown in figure 3.6, the growth rates of ES cells were similar, with a slight reduction in growth of the *Hdac1^{KO}; Hdac2^{Het}* (OHT treated) cells beyond day 2 when HDAC1 protein is lost and the level of HDAC2 is decreased. This result suggests that homozygous deletion of *Hdac1*, heterozygous deletion of *Hdac2* had a little effect on the growth rate.

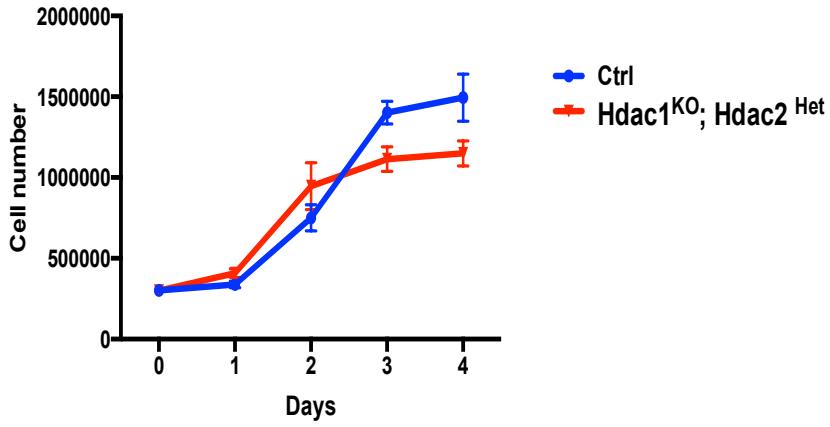


Figure 3.6: proliferative capacity of *Hdac1$^{Lox/Lox}$*; *Hdac2$^{Lox/WT}$*; *CreER* ES cells is unchanged. Growth rate of untreated (Ctrl) and *Hdac1^{KO}; Hdac2^{Het}* cells (OHT-treated) following gene inactivation was assessed by counting cells over a 4-day period. All values are means (n = 3) ±SEM.

Next, the ability of *Hdac1*^{KO}*; Hdac2*^{Het} cells to retain pluripotency when cultured in the presence of LIF was assessed and their ability to differentiate upon removal of LIF. Control (untreated) and *Hdac1*^{KO}*; Hdac2*^{Het} (OHT-treated) cells were plated at low density in the presence or absence of LIF, cultured for six days and then assayed for alkaline phosphatase (AP) activity, a pluripotent marker. We observed that, colonies derived from control and *Hdac1*^{KO}*; Hdac2*^{Het} cells had equal AP staining indicating that they were able to retain pluripotent in the presence of LIF and were able to differentiate upon withdrawal of LIF (Figure 3.7). *Hdac1*^{KO}*; Hdac2*^{Het} cells showed an increased percentage of mixed colonies in the presence of LIF which suggested a small increase in differentiated cells (increased by approximately 20%). In the absence of LIF, controls and *Hdac1*^{KO}*; Hdac2*^{Het} cells showed a comparable level of differentiated colonies (Figure 3.7). Overall, these data demonstrated that the proliferation and differentiation capacity of *Hdac1*^{KO}*; Hdac2*^{Het} cells is not inhibited, KO cells are able to proliferate in the presence of LIF and able to differentiate upon removal of LIF.

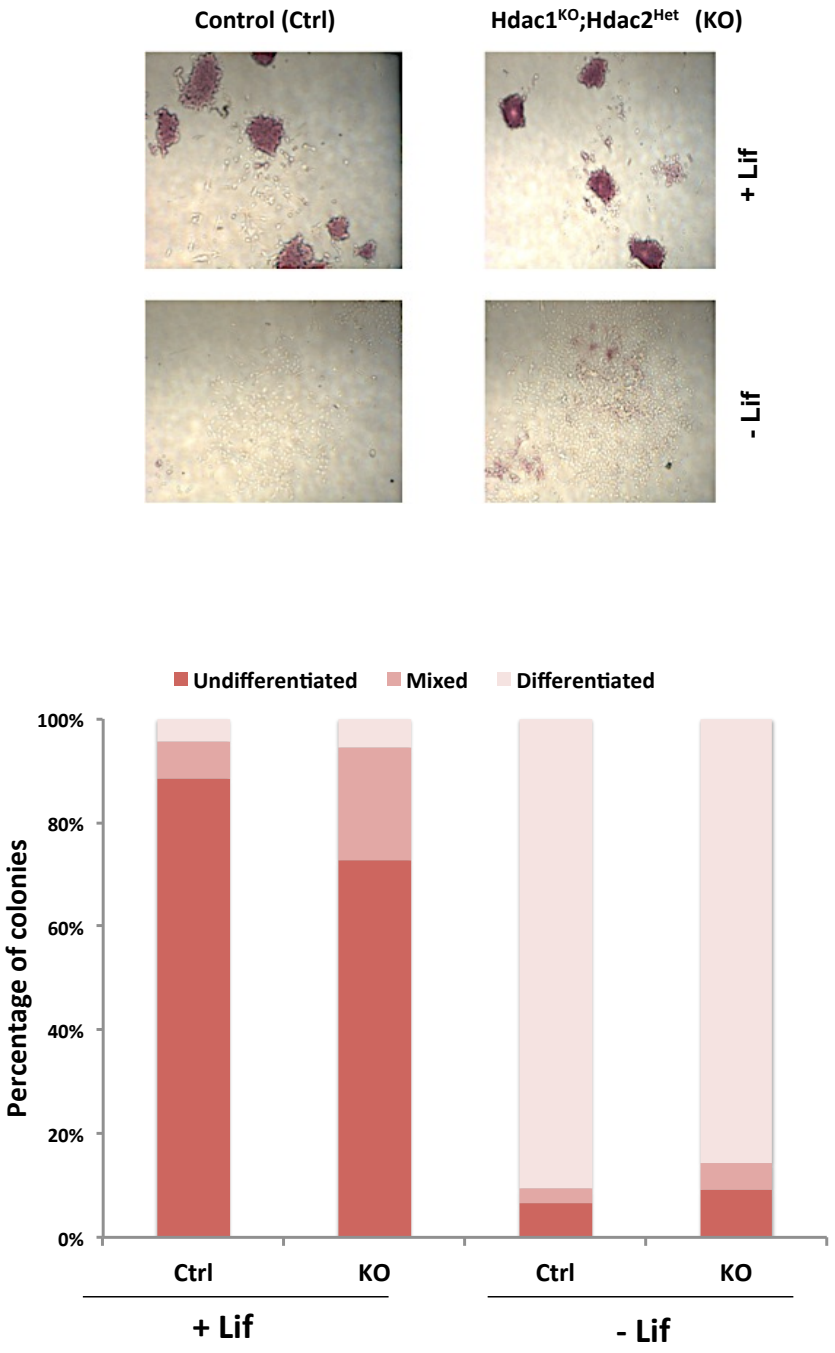


Figure 3.7: *Hdac1*$^{Lox/Lox}$; *Hdac2*$^{Lox/WT}$; CreER ES cells are able to differentiate upon LIF withdrawal. ES cells were cultured at low density in the presence (+) or absence of LIF for 6 days before staining for the presence of alkaline phosphatase and visualised by light microscopy. Colonies were scored undifferentiated (dark purple staining), mixed (intermediate purple staining) and differentiated (colorless).

The fact that we observed a very slight reduction in the growth rate of *Hdac1*^{KO}*; Hdac2*^{Het} cells beyond day2 compared to control, this correlates with a reduction in the protein levels of HDAC1 and HDAC2. Therefore, the cell cycle profile of *Hdac1*^{KO}*; Hdac2*^{Het} cells was analyzed compared to control cells over an 6-day period. As observed in figure 3.8, both control and *Hdac1*^{KO}*; Hdac2*^{Het} cells displayed similar cell cycle profile. Although, we detected a slight increase in the percentage of cell death in *Hdac1*^{KO}*; Hdac2*^{Het} cells, by day 6, 13.8% of *Hdac1*^{KO}*; Hdac2*^{Het} cells showed a sub-G1 DNA content compared to 4.2% of control cells (Figure 3.8). This result may explain the difference in the growth rate we observed in KO cells which may indicate that KO cells are more susceptible to cell death (Figure 3.6).

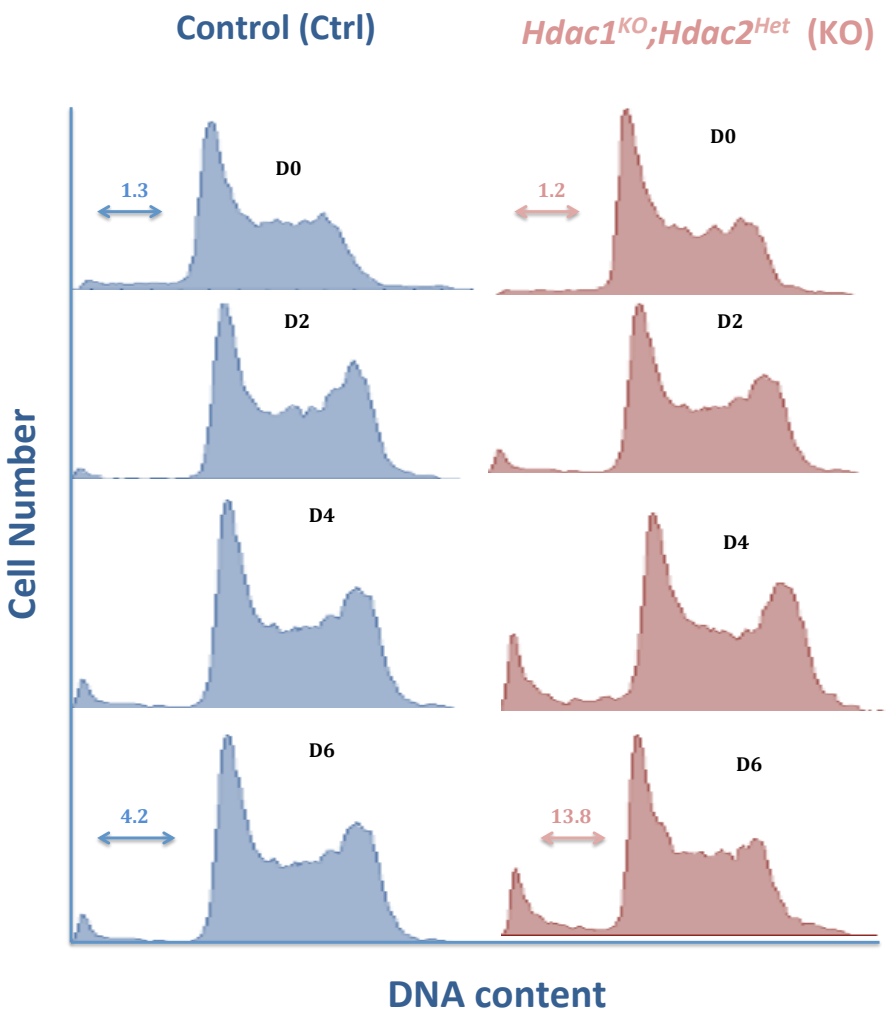


Figure 3.8: Cell cycle analysis of Hdac1$^{Lox/Lox}$; Hdac2 $^{Lox/WT}$; CreER ES cells shows an increase in the percentage of sub-G1 cells. Cell cycle distribution of Ctrl (untreated) and *Hdac1^{KO}; Hdac2^{Het}* (OHT-treated) ES cells over a 6-days period was performed using propidium iodide (PI) staining and FACS analysis. The arrow indicates the percentage of cells with a sub-G1 amount of DNA.

3.2.5 Gene expression profiling of *Hdac1$^{Lox/Lox}$; Hdac2$^{Lox/WT}$; CreER* ES cells in LIF-free media.

Loss of HDAC1 altered the differentiation capacity of ES cells (Dovey et al, 2010), Therefore to analyze differentiation of *Hdac1^{KO}; Hdac2^{Het}* ES cells, the transcriptome of ES cells was examined in LIF-free media. LIF maintain ES cell self-renewal via binding to the gp130 receptors and activation of the STAT3 signaling pathway; withdraw of LIF is a subtle method of inducing differentiation in ES cell.

RNA was isolated from control (untreated) and *Hdac1^{KO}; Hdac2^{Het}* (OHT-treated) cells that were cultured in the presence and absence of LIF for four days and then used to perform a comparative microarray analysis using an Illumina Whole-Genome Expression BeadChip platform. We compared the transcriptome of control (C+) and *Hdac1^{KO}; Hdac2^{Het}* KO (K+) cells cultured in the presence of LIF and control (C-) and *Hdac1^{KO}; Hdac2^{Het}* KO (K-) cells cultured in the absence of LIF. Quality control of total mRNA was performed using a 2100 Bioanalyser (Agilent), samples that had an RNA integrity number (RIN) of 8.6 or higher were selected for processing and array hybridization.

Transcripts up- or down-regulated by ≥1.4-fold (FC ≥1.4, adjusted $P<0.05$) were identified from three independent experiments using ArrayTrack analysis software. A total of 470 transcripts were deregulated in KO compared to control (C+ versus K+), with 337 up-regulated and 133 down-regulated transcripts (Figure 3.9A and appendix table 4), which are considered as the effect of *Hdac1^{KO}; Hdac2^{Het}* deletion in ES cells transcriptome. The large number of up-regulated transcripts compared to down-

regulated is consistent with the role of HDAC1 and HDAC2 in transcription repression, and also the detected number of down-regulated genes suggested their role in transcriptional activation at specific genes. Removing of LIF resulted in a change in the expression of 1,081 transcripts (C+ versus C-), with approximately the same numbers of up-(577) and down-regulated genes (504) . Finally, we compared control and KO cells cultured in the absence of LIF (C- versus K-), and we found 553 deregulated genes, of which 330 were up-regulated and 223 down-regulated (Figure 3.9A and appendix table 4).

Hierarchical clustering of samples based on their signal detection suggests that the transcription programme of samples (control and KO) cultured in the presence of LIF is distinct from the one that cultured in the absence of LIF (Figure 3.9B).

A. **B.**

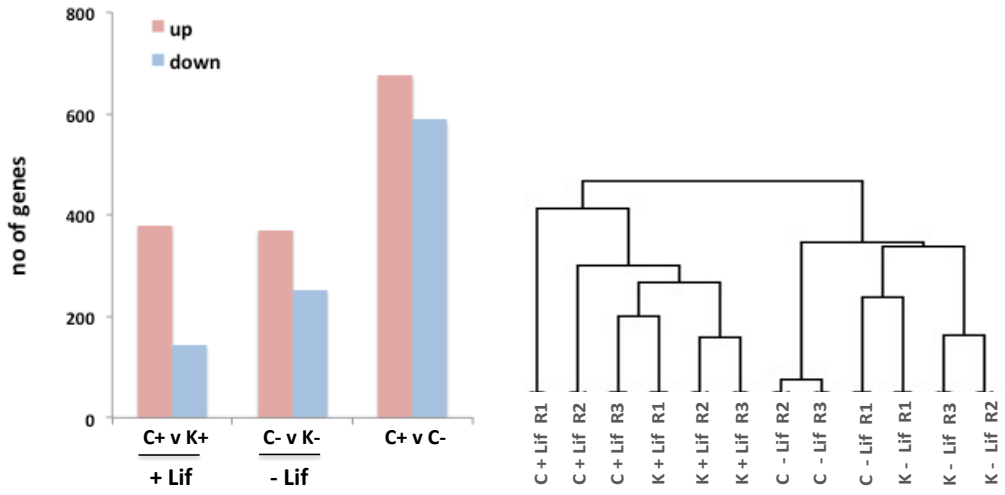


Figure 3.9: Gene expression profiling of *Hdac1*$^{Lox/Lox}$; *Hdac2*$^{Lox/WT}$; *CreER* ES cells. Control untreated (C) and Knockout KO (K) ES cells were cultured for four days in the presence of LIF (C+),(K+) or in the absence of LIF (C-),(K-). (A) The number of differentially expressed genes altered by ≥ 1.4-fold change (adjusted P< 0.05) is shown. (B) Hierarchical clustering of samples based on signal detection values. Hybridisation experiments were performed in triplicate using mRNA that had an RNA integrity number of 8.6 or higher.

Next, the expression level of genes associated with pluripotency and differentiation of ES cells were analyzed in samples from the microarray results (Figure 3.10). In a comparative analysis, we observed that, expression of pluripotent genes was down-regulated in control cells in the absence of LIF (C+ versus C-), as expected upon removal of LIF. Interestingly, a slight reduction on the same set of genes was also observed in KO cells cultured in the presence of LIF (C+ versus K+). Moreover, removal of LIF in *Hdac1*^{KO}*; Hdac2*^{Het} cells caused an increased reduction in the expression of pluripotent genes compared to Control (C- versus K-) (Figure3.10). In terms of differentiation specific markers (appendix table 3), we observed a slight increase in the expression of genes associated with differentiation in the absence of LIF (C+ versus C-) (Figure 3.10). However, KO cells that were cultured in the absence of LIF did not show similar induction in the expression of differentiation genes (Figure 3.10).

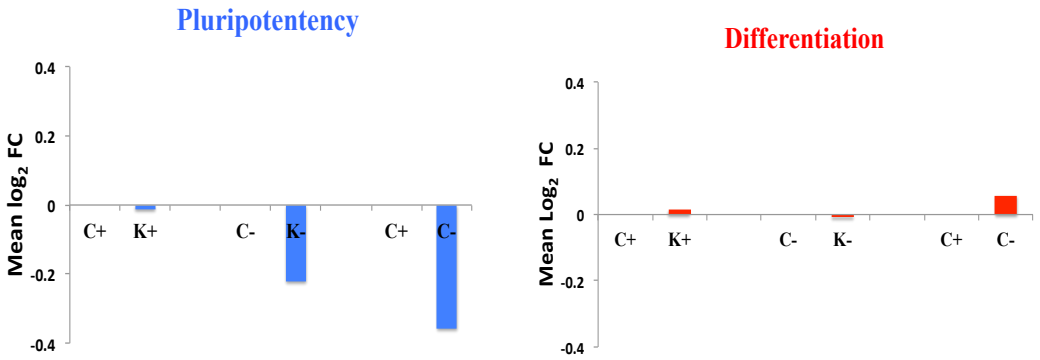


Figure 3.10: Comparative analysis of pluripotency and differentiation genes in *Hdac1*$^{Lox/Lox}$*; Hdac2*$^{Lox/WT}$*; CreER* ES cells. Analysis of expression levels of pluripotency and differentiation associated genes between Control, untreated (C+) and KO (K+) in the presence of LIF, with Control (C-) and KO (K-) in the absence of LIF, and between (C+) and (C-), using mean $\log_2$ fold change of microarray data.

To verify the microarray results, the expression levels of a set of genes were validated by quantitative real-time PCR (qRT-PCR). For each of the genes we analyzed we were able to corroborate the microarray result (Figure 3.11). The transcript level of pluripotent factor *Nanog*, was 1.5-fold down-regulated on the array and 2.6-fold by qRT-PCR, in control compared to KO (in the presence of LIF). In addition, *Oct4 (Pou5f1)* was down regulated by 1.4-fold. The transcript level of *Amnionless* and *Blvrb* were 2.6 and 1.8-fold up-regulated on the array and 5.1 and 3.9-fold by qRT-PCR, respectively (in the presence of LIF). As observed previously in microarray results, transcription levels of pluripotent markers were reduced in KO more than control (in the absence of LIF), Nanog and Oct4 being reduced 1.6-fold and 1.7-fold by qRT-PCR, respectively (Figure 3.11). These data suggest a positive role of HDAC1and HDAC2 in the expression of embryonic stem cell pluripotent markers.

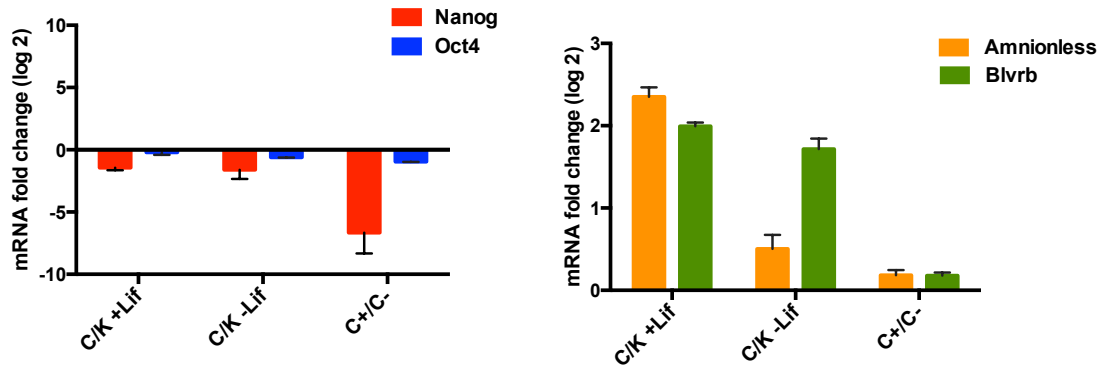


Figure 3.11: Validation of changes in gene expression by qRT-PCR. Validation of the changes in expression of some genes from the microarray by qRT-PCR, normalized to GAPDH. Experiments were performed in triplicate (n=3), mean values ± SEM plotted.

Then analysis of functionally related genes groups among deregulated genes was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID). In the presence of LIF (control compared to KO) , we observed enrichment of genes involved in transcription regulation among down-regulated genes ($p = 2.9 X10^{-5}$) (Figure 3.12). Down-regulated transcripts were also enriched for genes with role in regulation cell cycle and RNA processing ($p =1.4 \times 10^{-2}$, 7.7×10^{-3}), which suggests a role for HDAC1/2 in these processes. In the absence of LIF (control compared to KO), among up-regulated transcripts we found a significant enrichment for genes associated with embryonic development ($p = 4.2 \times 10^{-5}$), and genes that involved in regulation of cell cycle were down-regulated ($p = 6.5 x10^{-3)}$ as might be expected (Figure 3.13). In $Hdac1^{KO}$; $Hdac2^{Het}$ cells (KO) cultured in the absence of LIF, we observed a down-regulation of genes associated with nucleosome assembly, RNA processing and regulation of cell proliferation ($p = 3.3x10^{-9}$, $5.2x10^{-3}$ and 9.2x10-^{3}, respectively) (Figure 3.14).

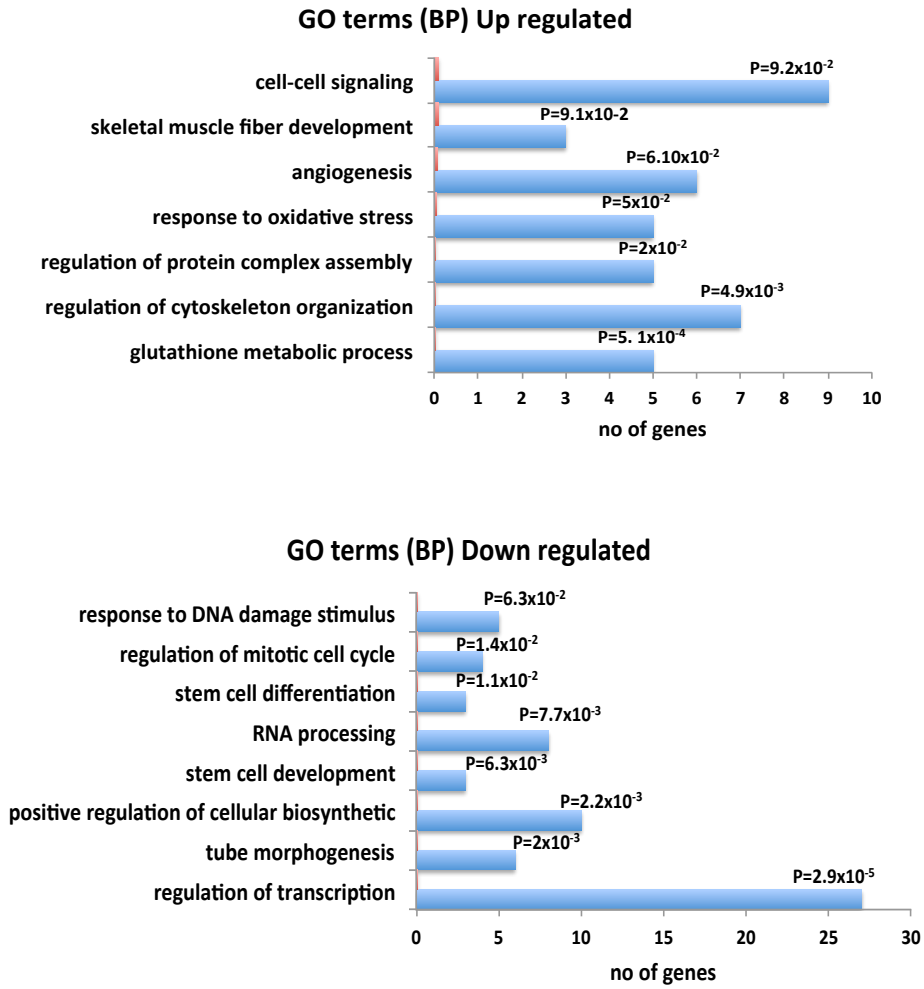
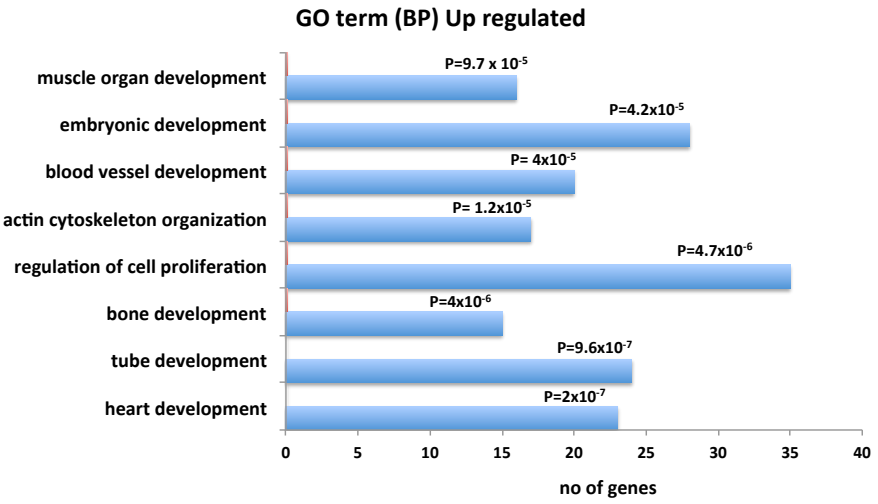


Figure 3.12: Functional annotation clustering of differentially expressed genes between control (untreated) and KO (OHT-treated) in the presence of LIF. Biological process gene ontology terms (BP-GO terms) of the up-regulated and down-regulated genes were identified in ES cells using DAVID. Analysis reveals enrichment of genes involved in RNA processing and stem cell differentiation are down-regulated and cell signaling genes are up-regulated.



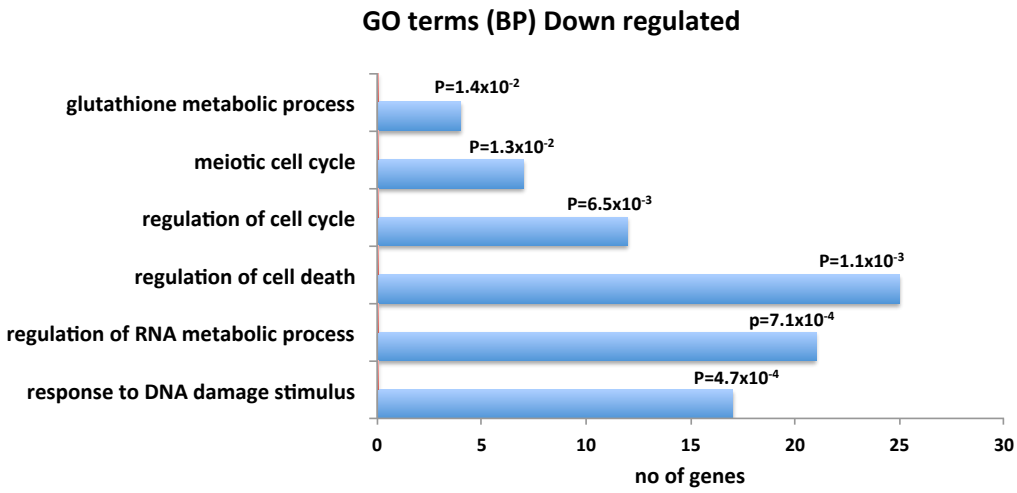


Figure 3.13: Functional annotation clustering of differentially expressed genes between control (untreated) in the presence of LIF (C+) and control in the absence of LIF(C-). Biological process gene ontology terms (BP-GO terms) of the up-regulated and down-regulated genes were identified in ES cells using DAVID. Analysis reveals enrichment of genes involved in stem cell development are up-regulated and regulation of cell cycle genes are down-regulated

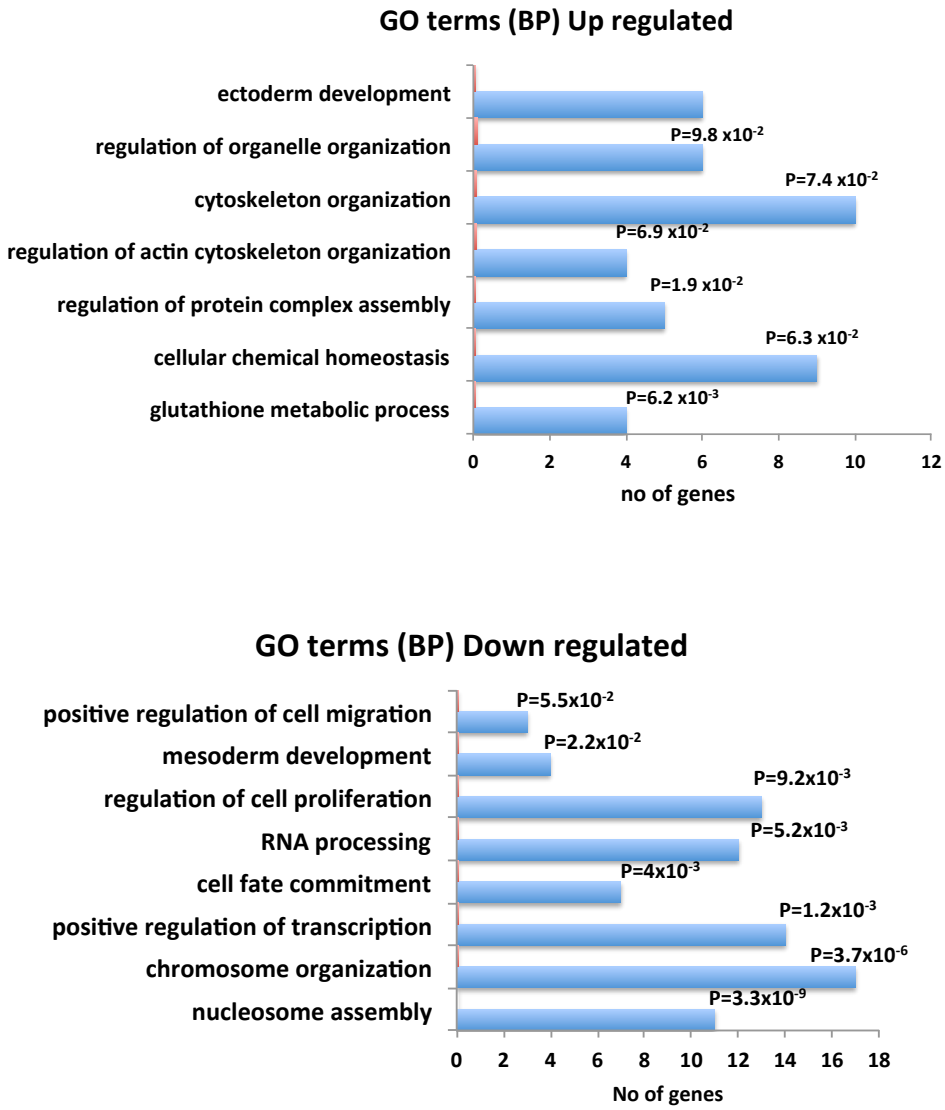


Figure 3.14: Functional annotation clustering of differentially expressed genes between control (untreated) and KO (OHT-treated) in the absence of LIF. Biological process gene ontology terms (BP-GO terms) of the up-regulated and down-regulated genes were identified in ES cells using DAVID. Analysis reveals enrichment of genes involved in stem cell differentiation are up-regulated and cell proliferation genes are down-regulated.

3.3 Conclusions

Inducible inactivation of *Hdac1*^{KO}*; Hdac2*^{Het} cells does not inhibit proliferation or differentiation ability of ES cells. KO cells were able to proliferate in the presence of LIF and exit pluripotent state upon LIF withdrawal (Figure 3.7). *Hdac1*^{KO}*; Hdac2*^{Het} cells had a reduction in the expression level of pluripotent factors, which is more pronounced upon removal of LIF suggesting a positive role for HDAC1 and HDAC2 in the maintenance of ES cells pluripotency (Figure 3.10). We also observed a reduction in the stability of co-repressor complexes (Figure 3.4). Analysis of a biochemical deacetylase activity of KO cells indicates significant reduction (≈ 56%) in the total activity, which reveals the effective role of HDAC1/2 in ES cells. Analysis of histone acetylation reveals an increased in the acetylation status of analyzed histone with the largest change in H3K56Ac (Figure3.5).

Chapter 4: Histone deacetylase (HDAC) 1 and 2 are essential for accurate cell division and pluripotency of embryonic stem cells

4.1 Chapter aims

As discussed in chapter three, the viability and pluripotent potentials of *Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/wt}$;CreER ES cells was unaffected. It has been previously demonstrated in other system (LeaBoeuf, M., et al., 2010, Yamaguchi, T., et al, 2010 and Dovey, O.M., et al, 2013) that deletion of both HDAC1 and HDAC2 is required to produce a phenotype, which suggests that the function of HDAC1/2 in many cell type is redundant. Therefore, to circumvent this functional redundancy, we generated a double conditional knockout (**DKO**) *Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/Lox}$; CreER ES cell line. Using DKO ES cells, I aimed to assess the biochemical and proliferative properties of ES cells lacking HDAC1/2 and their contribution to the regulation of the ES cell transcriptome.

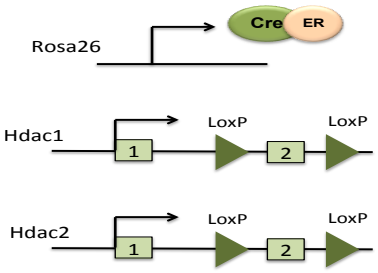
4.2 Results

4.2.1 Generation of conditional double-knockout (DKO) ES cells

An E14 ES cell line expressing a Cre/estrogen receptor (CreER) fusion from the ROSA26 locus was used to generate *Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/Lox}$; CreER DKO ES cell line, in which exon 2 of each gene is flanked by LoxP sites (Figure 4.1A). Adding 4-hydroxytamoxifen (OHT) to the growth media induces Cre-recombinase activity and resulting in a deletion of exon 2 which disrupts the open reading frame of HDAC1 and HDAC2 and a premature stop codon is introduced into exon3. mRNA is subjected to nonsense-mediated decay and/or a non-functional protein is produced due to lack the catalytic deacetylase domain in both HDAC1and HDAC2.

Inactivation of *Hdac1* and *Hdac2* genes resulted in loss of each protein 2-3 days following OHT treatment (Figure 4.1B,C), indicating that the half-life of each protein is ≈ 24 hours.

A.



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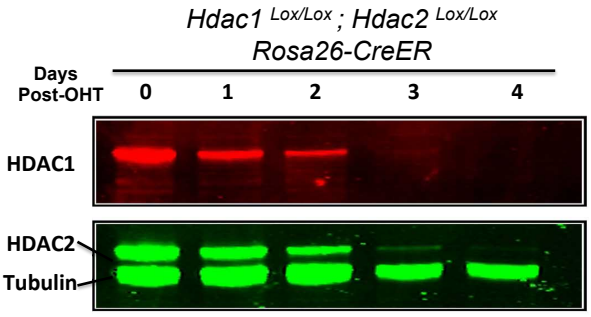

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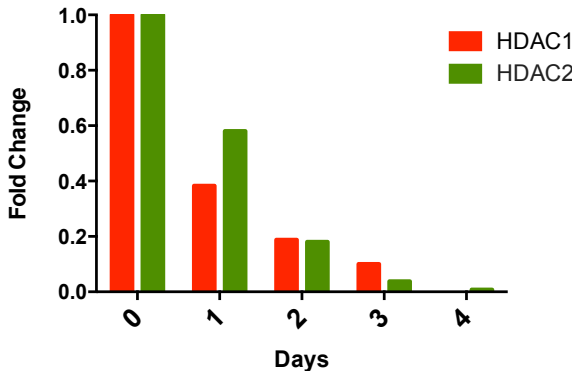


Figure 4.1: Generation of conditional double-knockout (DKO) ES cells. (A) Schematic diagram of the model system. An E14 ES cell line constitutively expressing a Cre/estrogen receptor (CreER) fusion from the ROSA26 locus was used to generate homozygous conditional knockout alleles for both Hdac1 and Hdac2. Both genes are inactivated by deletion of exon 2,which is flanked by LoxP sites. (B) Quantitative Western blot showing loss of HDAC1 and HDAC2 proteins following gene inactivation (0-4 d). Cells were cultured in the presence of 4-hydroxytamoxifen (OHT) for 24h to induce the deletion of Hdac1/2.α-Tubulin was used to normalize protein loading, and blots were visualized and quantified using an Odyssey scanner. (C) Quantitative Western blotting was used to quantify the change in HDAC1 and HDAC2 proteins following gene inactivation (0-4 d). Fold change in protein levels relative to α-Tubulin. Data are representative of n>3 independent experiments.

Next, the total deacetylase activity of the cell was measured on four consecutive days following 4-OHT treatment. Loss of HDAC1 and HDAC2 resulted in a 60% decrease in cellular deacetylase activity (Figure 4.2), which indicates that HDAC1/2 are biochemically the predominant HDAC enzymes in the ES cells.

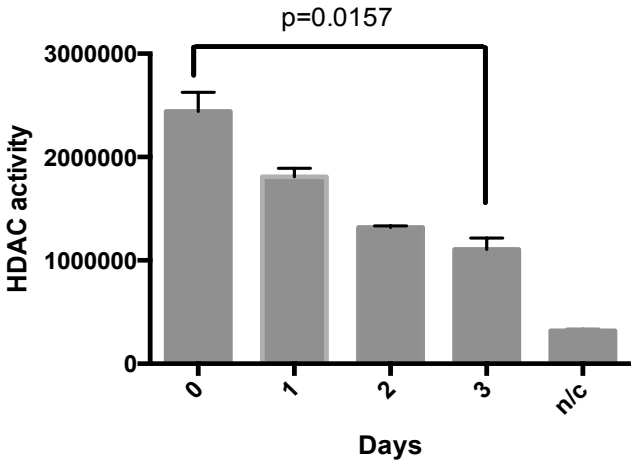


Figure 4.2: Deletion of HDAC1/2 results in reduction in Deacetylase activity. Total deacetylase activity was measured in whole-cell extracts on 4 consecutive days following OHT treatment; n/c represent the negative control. Data are representative of n>3 independent experiments. Significant (P value) was calculated using a two-tailed t test.

4.2.2 Inactivation of *Hdac1/2* causes loss of cell viability

Analysis of the growth ability and cell cycle profile of *Hdac1/2* deleted ES cells compared to the control (untreated) cells revealed a loss of cell viability in the absence of HDAC1/2. We monitored the growth of DKO ES cells compared to control (untreated) cells and found that DKO cells stopped proliferating beyond day 2, followed by a profound loss of cell viability at day 4 (Figure 4.3A). Three days following OHT treatment, we observed a change in the morphology of the DKO cells compared to controls (Figure 4.3B). In order to assess the effect of *Hdac1/2* deletion on cell cycle distribution, the cell cycle profile of DKO cells were analyzed over four days using propidium iodide (PI) staining followed by FACS. As shown in figure 4.3C, the percentage of cells with a sub-G1 content (indicative of cell death) increased form 2% to 11% by day 3, and to 75% at day4 in DKO cells.

Loss of HDAC1 has previously been implicated in reduced proliferation in ES cells (Lagger, M., et al. 2002). Moreover, loss of cell proliferation is a common phenotype in all *Hdac1/2* knockout and knockdown studies (Yamaguchi, T., et al., 2010; Wilting, R.H., et al., 2010, and Zupkovitz, G., et al., 2010), which is associated with up-regulation of cyclin-dependent kinase (CDK) inhibitors $P21^{WAF1/CIP1}$ and $P57^{Kip2}$ leading to G1 phase arrest. However, our cell cycle analysis revealed no obvious cell cycle arrest before cell death on day 4.

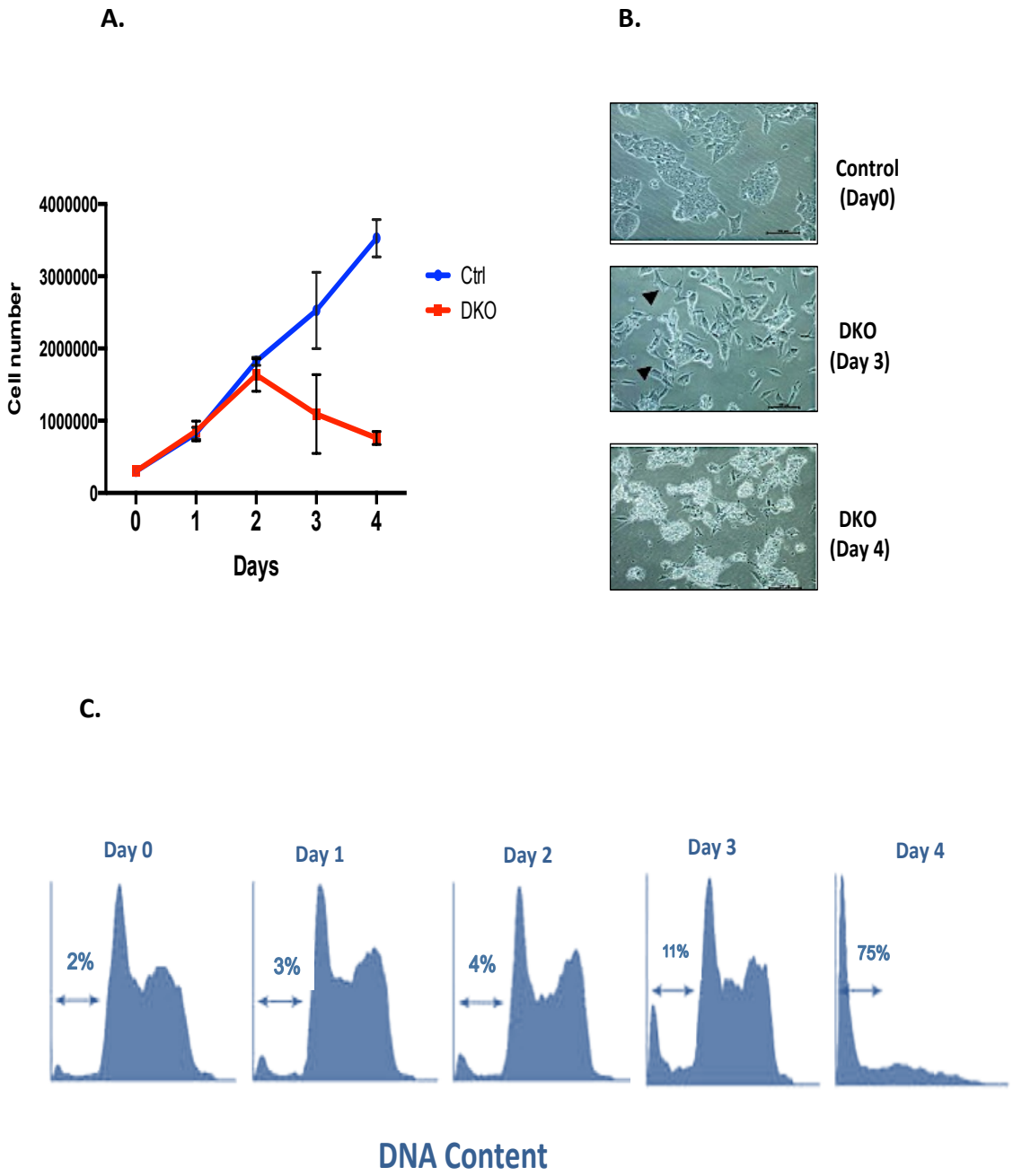


Figure 4.3: Inactivation of *Hdac1/2* causes loss of cell viability. (A) Comparative viable cell counts between control ES cells (Ctrl, untreated DKO cells) and Hdac1/2 double-knockout ES cells. All values are means (n=3) ± SEM (B) Phase contrast microscopy used to take images of Hdac1/2 deleted cells at the indicated time points following 4-hydroxtamoxifen (OHT) treatment. The black arrows indicate examples of cells that have undergone a change in morphology. (C) Cell cycle distribution of Hdac1/2 deleted cells over a 4-d period following gene inactivation was performed using propidium iodide staining and FACS analysis. The arrow indicates the percentage of cells with a sub-G1 amount of DNA.

4.2.3 Cell death in DKO ES cells is mediated by apoptosis

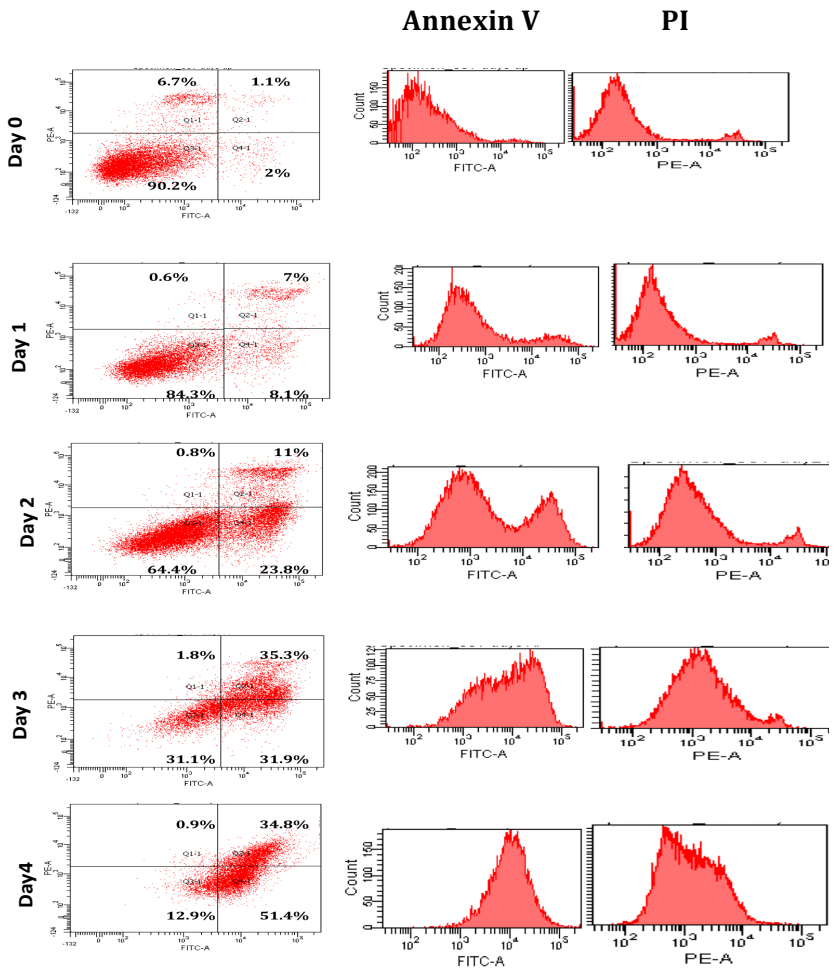
We previously observed that inactivation of *Hdac1/2* causes loss of ES cells viability. Therefore, to determine whether DKO ES cell were dying via apoptosis, flow cytometry was performed in DKO ES cells using AnnexinV and Propidium iodide (PI) staining.

One of the earlier cellular changes of the apoptotic process is translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the cell surface. Annexin V is a protein that interacts strongly with exposed phosphatidylserine, when it is fluorescently labelled, it can be used to detect apoptotic cells. Propidium iodide (PI) is a fluorescent molecule that binds nucleic acid. PI is used in conjunction with Annexin V, which allows for the discrimination between viable, apoptotic and necrotic cells depending on their plasma membrane integrity and permeability. The intact plasma membrane excludes PI in viable and early apoptotic cells, whereas, the membrane of late apoptotic and necrotic cells are permeable to PI.

To confirm whether *Hdac1/2* deleted cells were dying via apoptosis, DKO cells were stained with Annexin and PI at days 0, 1, 2, 3 and 4 then analyzed using flow cytometery. As shown in figure 4.4A (lower left quadrant), the percentage of viable cells (Annexin V / PI negative) decreased from 90.2% to 12.9% at days 4. A total of 51.4% of the cells were positive for Annexin V and excluded PI, which represents early apoptotic cells.

The late apoptotic cell population increased gradually from 1.1% to 34.8% at day4 (upper right quadrant). The AnnexinV/ PI staining result suggests that, cell death in DKO cells is mediated by apoptosis. To further confirm this we performed Western blots for apoptotic markers on protein extracts from DKO cells (0-3 days) following gene inactivation. Caspase3 plays a central role in the process of apoptosis, it synthesized as an inactive pro-enzyme that is activated by proteolytic cleavage during apoptosis. Induction of apoptosis can be followed by monitoring the expression levels of full length (32 kDa) pro-caspase 3 as well as the large fragment (19 kDa) of active caspase-3 generated by cleavage at aspartic acid 175. As seen in figure 4.4 B, we detect increased in the level of active subunit of caspase 3 by day3 following gene inactivation. During apoptosis, active caspase3 proteolytically cleaves and inactivates Poly (ADP-ribose) polymerase 1 (PARP). It can be seen that over the 3-day time period, the protein level of full length PARP (116 kDa) was reduced in parallel with the increased in the level of cleaved large fragment (89 kDa) of PARP (figure 4.4B). These result show that deletion of HDAC1/2 in ES cells resulted in cells dying via apoptosis.

A.



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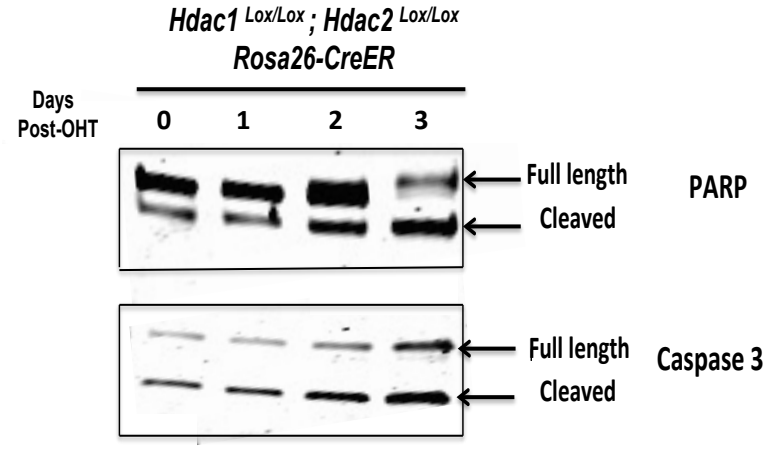
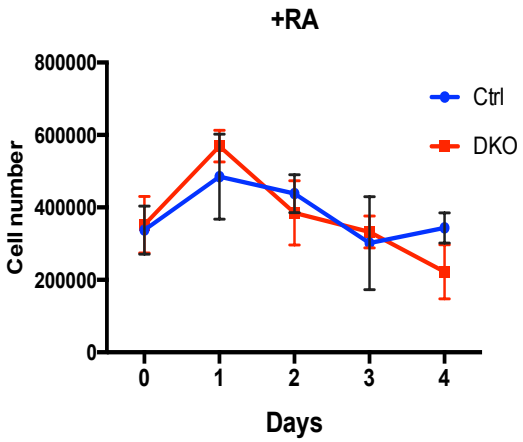


Figure 4.4: Cell death in DKO cells is mediated by apoptosis. (A) Evaluation of apoptosis by AnnexinV/PI dual staining assay and flow cytometer analysis of *Hdac1/2* deleted cells over a 4-day period following gene inactivation. (B) Quantitative Western blot showing expression of PARP and Caspase3 protein levels following gene inactivation (0-3days). Blots were visualized and quantified using an Odyssey scanner.

4.2.4 Cell cycle exit rescues the viability of DKO ES cells

The predominant phenotype of *Hdac1/2* DKO cells is a loss of viability 4 days after gene inactivation. HDAC1/2 have been implicated in the regulation of cell cycle in a number of other model systems (Leggar, G., et al., 2002 and Zupkovitz, G., et al., 2010), therefore to test the effect of growth on the lethal phenotype we stimulated differentiation and cell cycle exit of DKO ES cells before deleting of HDAC1/2. The first differentiation method we used was the treatment of ES cells with retinoic acid (RA) for two days before the addition of OHT, after which we cultured the cells for four days in the absence of LIF. As shown in figure 4.5A, the majority of cells remained viable. Next, we stimulated differentiation of DKO ES cells by generating embryoid bodies (EBs). We also found that most of the DKO cells were able to aggregate to form EBs and remained viable after the deletion of *Hdac1/2* (figure 4.5B). These results demonstrate that, the lethal phenotype in the *Hdac1/2* deletion ES cells is cell cycle dependent.

A.



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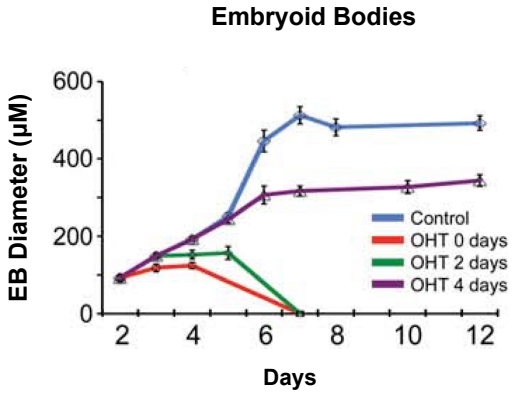


Figure 4.5: Cell cycle exit rescues the viability of the DKO ES cells. (A) ES cells were treated with 1µM retinoic acid (RA) for 2 days to induce differentiation and cell cycle withdrawal, before the addition of OHT. Comparative viable cell counts of untreated (Ctrl), and OHT-treated DKO cells are shown for a 4-days period. (B) Embryoid bodies (EBs) were generated by plating ES cells on to bacterial dishes . OHT was add to the growth media at the indicated times following plating. The mean size if EBs is shown (n>30) ± SEM. (Figure 4.5B is provided by Dr.Richard Kelly).

4.2.5 Loss of HDAC1/2 causes defective chromosomal segregation

The lethal phenotype observed in the DKO ES cells is dependent on an active cell cycle (Figure 4.5). Therefore, the next experimental direction was to search for the potential cell cycle defects in DKO cells. It has been previously observed that, deletion of other HDAC1/2 complex components, SDS3 and Sin3A, have a role in chromosome separation and segregation (David, G., et al. 2003; Silverstein, R., et al. 2003).

To explore the cell cycle defects, control (DKO cells, day0), individual *Hdac1*-KO and *Hdac2*-KO cells, a compound *Hdac1*-KO; *Hdac2*-Het KO, and DKO cells at day3 after gene inactivation, were stained with anti-α-tubulin, anti-γ-tubulin and Hoechst 33258 to visualize chromosomes during various stages of cell cycle (Figure 4.6).

It can be observed in figure 4.6A that the majority of DKO cells in metaphase had a monopolar rather than bipolar mitotic spindle. Additionally, we detected a significant increase in the number of DKO cells with segregation defects, which is not observed in the individual and a compound KO (Figure 4.6C,D). Interestingly, we observed a significant increase in DNA abnormalities with *Hdac1*-KO, *Hdac1*-KO; *Hdac2-Het* KO, and DKO cells, but not *Hdac2*-KO cells (Figure 4.6E), which correlate with the dosage of HDAC activity, in which all cell lines showed a reduction in the HDAC activity but not *Hdac2*-KO cells (Figure 4.7).

Segregation defects, including lagging chromosomes and chromatin bridges, were only observed in the DKO cells, suggesting the presence of both pre-mitotic and mitotic errors. These results show that, loss of *Hdac1/2* causes mitotic errors and DNA replication defects. This may explained by data from Sirbue et al. (2011), who showed that HDAC1/2 are present at active replication forks. Moreover, it has been recently

shown that, inhibition or knockdown HDAC1/2 reduced the replication fork velocity and activates replication stress response (Bhaskara, S., et al., 2013). Therefore, these data supported our results that lethal phenotype in DKO ES cells is a combination of DNA replication and mitotic defects.

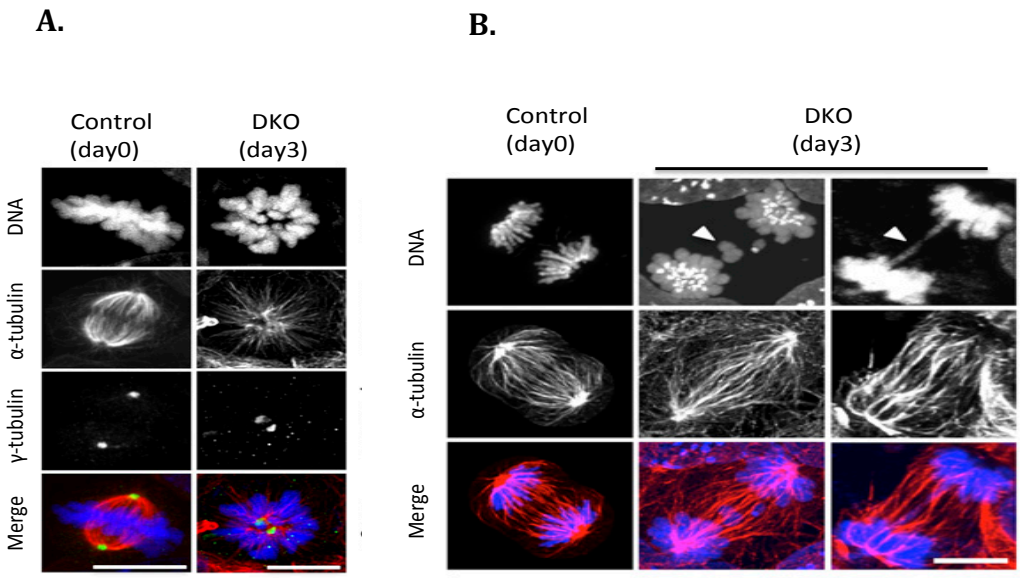


Figure 4.6: Loss of HDAC1/2 causes defective chromosomal segregation. ES cells were stained with anti-α-Tubulin (red), anti-γ-Tubulin (green), and Hoechst. Experiments were preformed on untreated DKO (day 0, control) and DKO cells following deletion (day 3), single *Hdac1* and *Hdac2* knockout cells and a compound *Hdac1*-KO; *Hdac2*-Het knockout cells. Images show examples of mitotic cells with monopolar spindles (A) and segregation defects (B) following *Hdac1/2* deletion. The white arrows indicate individual examples of lagging chromosomes (Center) and chromatin bridges (Right). The images correspond to z projections. (Scale bar, 10μM).

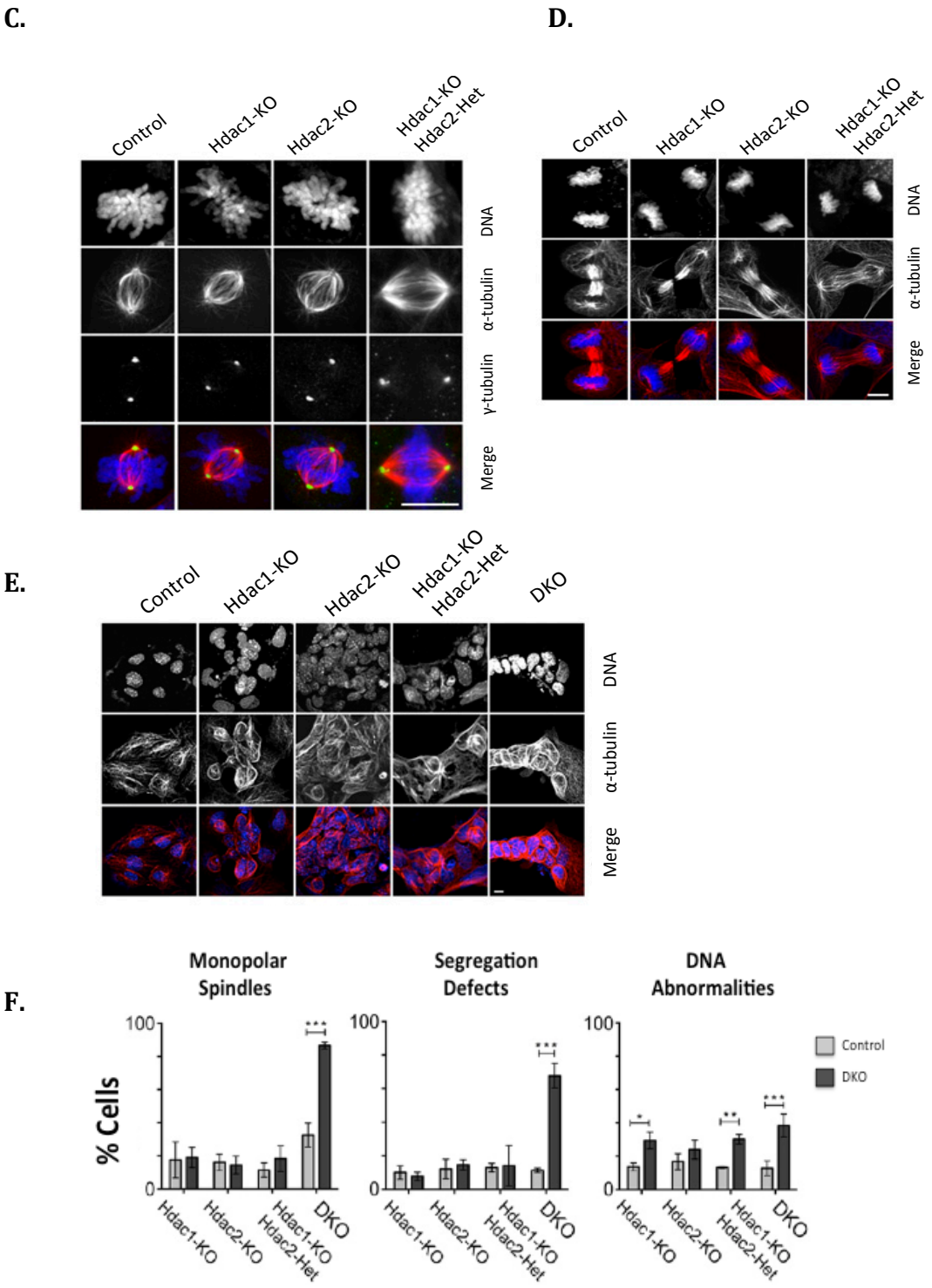


Figure 4.6 Continued. (C-D) Images show examples of individual mitotic and interphase (E) cells. (F) Quantitative analysis of chromosome segregation defects following loss of HDAC1/2. The mean (±SD) percentage of cells with abnormal DNA is indicated based on counts of at least 50 cells from n≥3 experiments. Significance (*p* value) was calculated using a two-tailed t test (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$). Images were provided by Laura O'Regan.

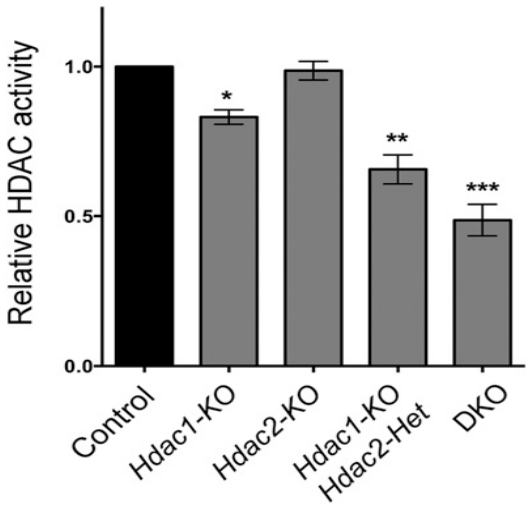


Figure 4.7: Total cellular deacetylase activity of cell lines . Deacetylase activity was measured from cells of the indicated genotype.All values are means (n>30) ± SEM and are normalized relative to the level of α-tubulin. Significance (*p* value) was calculated using a two-tailed t test (*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$).

4.2.6 Loss of HDAC1/2 disrupts corepressor complex integrity and leads to an increase in global histone acetylation

HDAC1 and HDAC2 are often found within a complex of proteins that are fundamental for their deacetylase activity and for binding DNA (Zhang ,Y. ,et al., 1999). HDAC1/2 are recruited into three main transcriptional corepressor complexes: Sin3A, NuRD and CoREST (Laherty,C., et al. 1997, Xue, Y., et al. 1998, You, A., et al. 2001). In order to assess the effect of HDAC1/2 deletion on the integrity of corepressor complexes, we performed Western blots on protein extracts from control and day3 DKO cells. As shown in figure 4.8 A and C, left panel, there is a reduction in the protein levels of Sin3A and MTA2, both are direct binding partners of HDAC1/2. We observed a 4.2 fold decrease in the level of Sin3A and a significant 7.1 fold decrease in the level of MTA2 which mediates the interaction of HDAC1/2 with the NuRD complex through its ELM2-SANT domain (Millard, C., et al. 2013, Lee, M., et al. 2006). The significant reduction we detected in the level of MTA2 can be explained by the fact that the ELM2-SANT domain of MTA1 wraps completely around the catalytic domain of HDAC1 making extensive protein-protein contracts (Figure 4.8E). Importantly, the conserved N-terminal region of ELM2 (residues 162–198), which binds an extended groove on the side of HDAC1, lacks extensive secondary structure. Therefore, in the absence of HDAC1/2 this region is likely to be solvent exposed and therefore lead to increased protein turnover of MTA2. We also observed a 2.6 fold reduction in the level of SDS3 that facilities the interaction of Sin3A/HDAC1 (David, G., et al 2003, Fleischer, T., et al. 2003). Interestingly, in the absence of HDAC1/2 we observed a 7-fold increase in the expression level of HDAC3 (Figure 4.8 B, D),

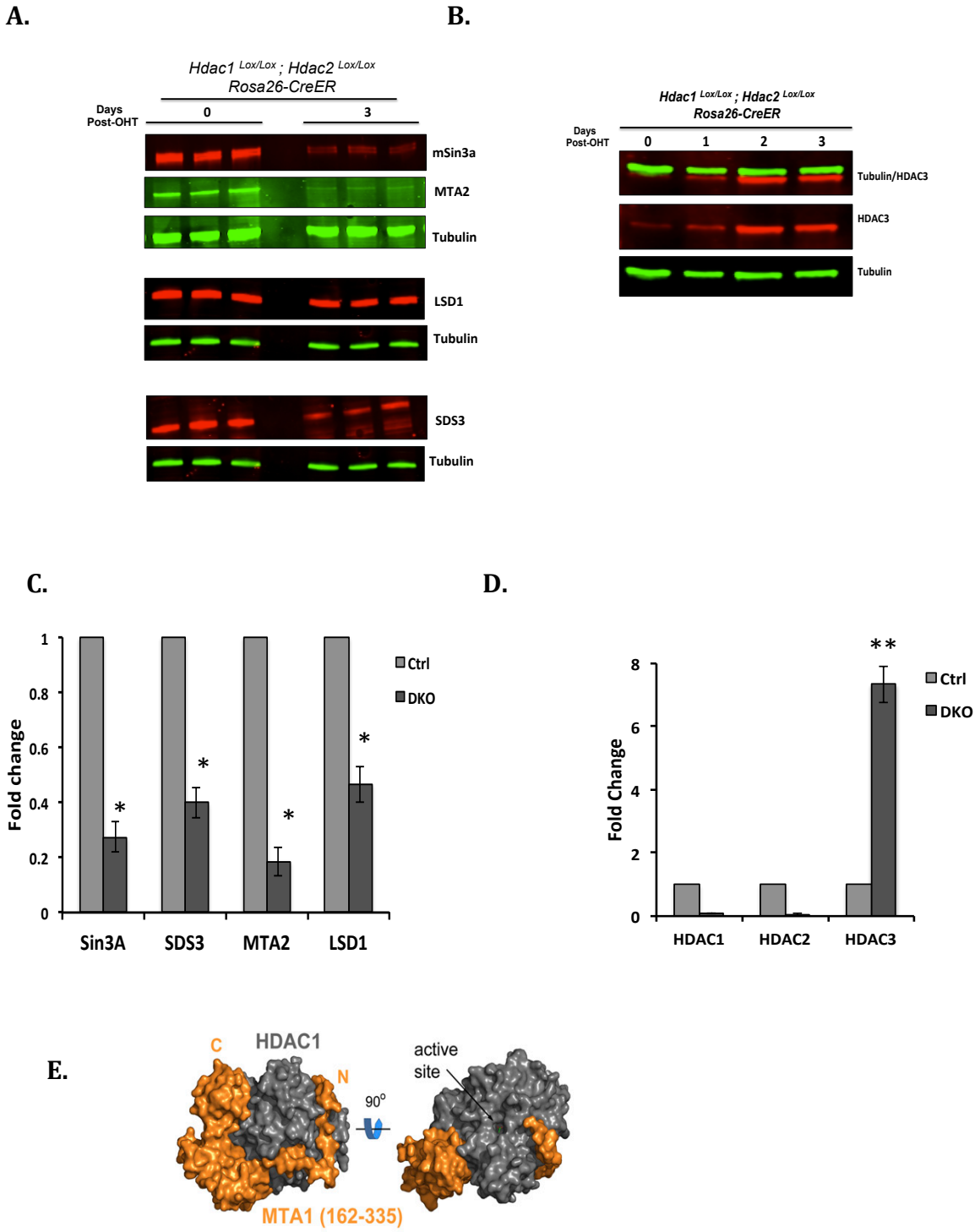


Figure 4.8: Loss of HDAC1/2 disrupts corepressor complex integrity. Experiments were performed on untreated (Ctrl), or OHT-treated double-knockout (DKO) cells 3d following gene inactivation. (A, B) Quantitative Western blot showing the relative levels of indicated proteins. (C, D) Quantitative western blot data for the indicated proteins were performed using an Odyssey scanner and normalized to the level of α-tubulin. Loss of HDAC1/2 causes increased expression of HDAC3 in DKO (day3) cells compared with control (Ctrl). (E) Structure of the ELM2-SANT region of MTA1 (orange) bound to the catalytic domain HDAC1 (grey). All values are means (n>3) ±SEM. The significance (*P* value) of data in C and D and was calculated using a two-tailed t test (**P* < 0.01, ***P* < 0.001) .

a highly related class I that a components of the SMRT/NCoR complex, which suggests a degree of compensation for the loss of HDAC1/2.

Next, global histone acetylation levels in the absence of HDAC1/2 were examined using quantitative Western blotting (Figure 4.9). We detected a relatively modest increase in acetylation levels at most sites due to the fact that pluripotent ES cells maintain a relatively plastic chromatin structure and consequently have high basal levels of histone acetylation (Dovey et al. 2010). Consequently, the notable changes detected were a 3-fold and 4-fold increased in the levels of H3K14Ac and H3K56Ac, respectively. Additionally, we observed that the levels of two methylation sites within H3 tail, H3K4me2 and H3K9me3, were unchanged. We conclude then, the loss of HDAC1/2 caused a reduction in the stability of the corepressor complexes that had an effect on the levels of global acetylation levels of H3 and H4.

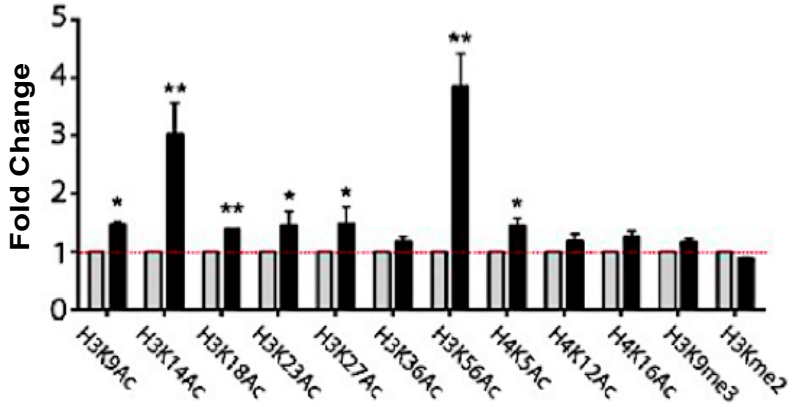


Figure 4.9: Deletion of HDAC1/2 leads to increased global histone acetylation. Quantitative Western blotting was used to determine the levels of global histone acetylation. Acetylation levels were normalized to the total amount of H3 quantified using an Odyssey scanner. All values are means (n>3) ±SEM. The significance (*P* value) of data was calculated using a two-tailed t test (*$P < 0.01$, **$P < 0.001$) .

4.2.7 HDAC1/2 regulate the ES cells transcriptome and are required for the expression of Oct4 and Nanog

HDAC1/2 have been implicated in the regulation of gene expression (Kelly, R. and Cowley, S., 2013). The global changes in histone acetylation in DKO ES cells (Figure 4.8) suggested that the pattern of gene expression may well be altered and therefore examined the consequence of *Hdac1/2* deletion on the ES cell transcriptome. mRNA was isolated from DKO cells at 0, 1, 2 and 3 days following *Hdac1/2* inactivation to perform a comparative microarray analysis using an Illumina Whole-Genome Expression BeadChip platform. Quality control of total mRNA was performed using a 2100 Bioanalyser (Agilent). Only samples that had an RNA integrity number of 8.6 or higher were selected for processing and array hybridization.

Transcripts that were deregulated ≥1.4-fold ($p < 0.05$) were identified from three independent experiments using ArrayTrack analysis software (Figure 4.10A and appendix table 5). Interestingly, we observed a correlation between the reduction in HDAC activity and the number of deregulated genes. We detected only three aberrantly expressed transcripts on day 1 and an increasing number on day 2 (560 genes) and 1,708 genes by day 3, as HDAC1/2 are progressively lost (Figure 4.10B). The majority of deregulated genes were up-regulated on day 2 (419 up-regulated compared with 141 down-regulated) and on day3 (994 up-regulated compared with 714 down-regulated), consistent with the role of HDAC1/2 in transcriptional repression. However, the large number of down-regulated genes also indicates the role of HDAC1/2 in transcriptional activation of specific genes, supported by recent genome-wide ChIP studies which revealed an enrichment of HDAC1 binding at active

gene loci (Wang, Z., et al. 2009, Kurdistanti, S., et al. 2002). To further verify the microarray results, the levels of six down-regulated, seven up-regulated, and five unchanged transcripts were quantified by quantitative real-time PCR (qRT-PCR) (Figure 4.10C). RT-PCR data for all 18 transcripts corroborated the microarray results.

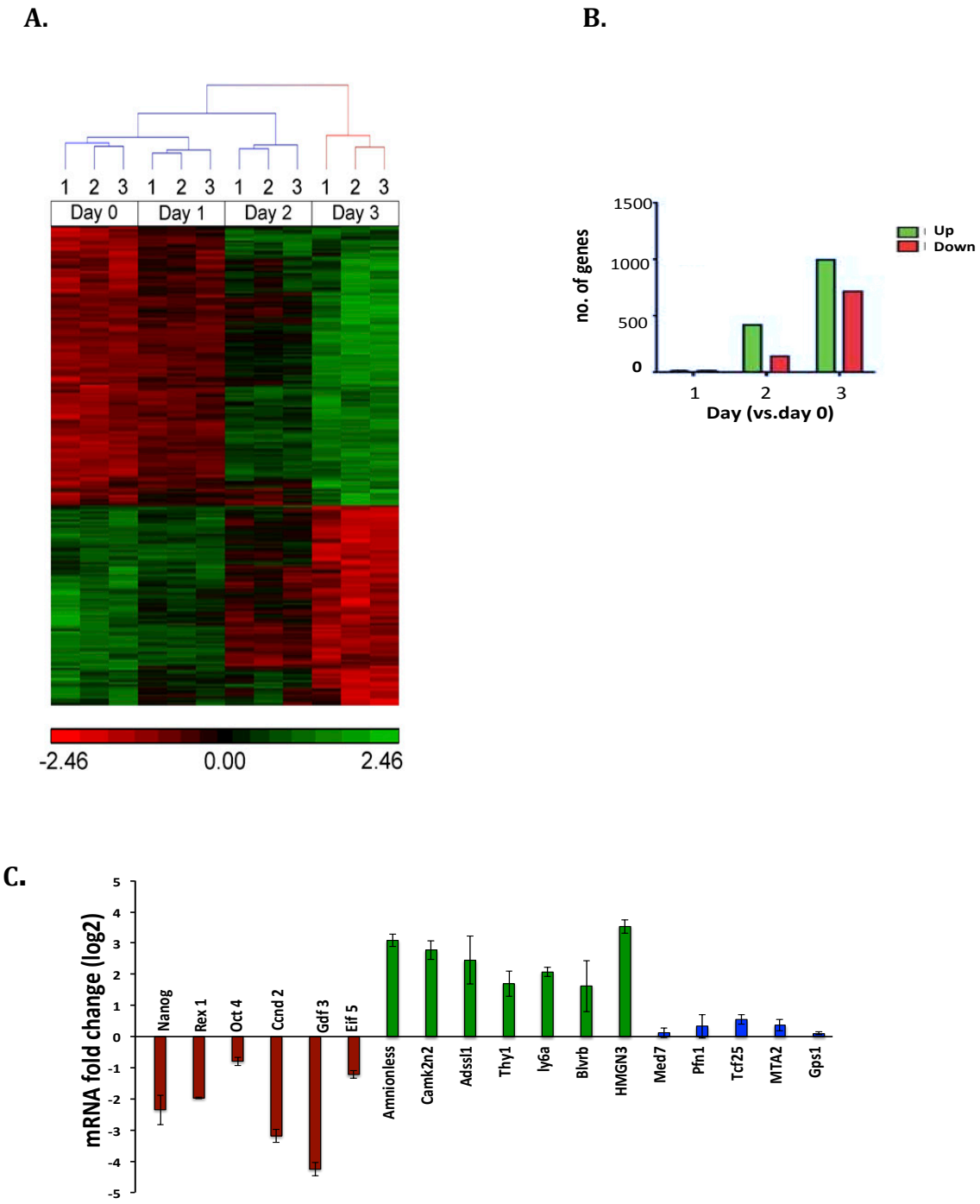


Figure 4.10: HDAC1/2 regulate the ES cell transcriptome. (A) A heat map showing 1,708 genes that are differentially expressed in DKO ES cells over a 3-d time course following gene inactivation. The red and green labeling indicates relative gene expression levels. (B) Number of genes differentially expressed at the indicated days (compared with day 0) following deletion of *Hdac1/2*. (C) qRT-PCR was used to validate the change in expression of a subset of genes from the microarray. Values indicate comparative means (n=3) ±SEM between DKO cells at day 0 and day 3 following gene inactivation.

We then performed an analysis of functionally related gene groups among deregulated genes using Database for Annotation, Visualization, and Integrated Discovery (DAVID). We found that there was an enrichment for genes involved in cell death are up-regulated, whereas cell cycle genes are down-regulated (Figure 4.11), confirming the phenotype observed in the DKO cells. Also, It can be observed that down-regulated transcripts are highly enriched for genes with a role in the regulation of transcription ($p = 7.4 \times 10^{-14}$) and cell cycle processes ($p = 4.88 \times 10^{-5}$). Interestingly, genes involved in RNA processing are also decreased to the same level of significance ($p = 8.4 \times 10^{-14}$), suggesting a putative role for HDAC1/2 in the regulation of RNA splicing.

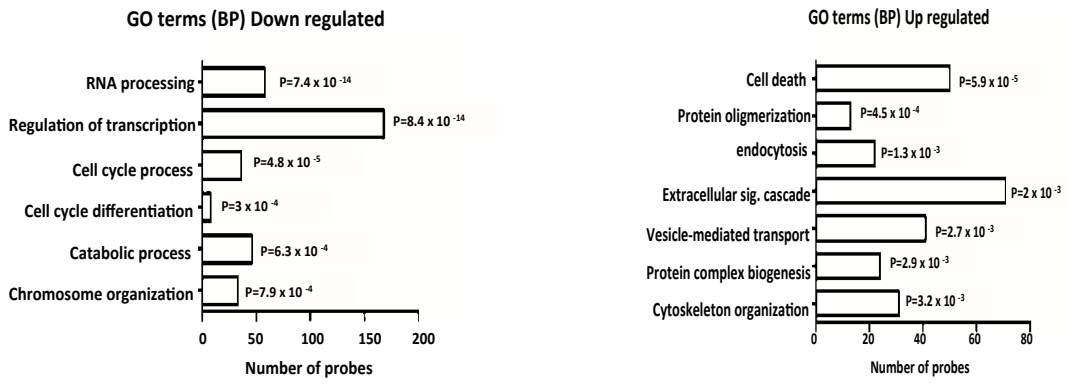


Figure 4.11: Functional annotation analysis of differentially expressed genes. DAVID was used to identify biological process (BP) and gene ontology (GO) of probes up-regulated or down-regulated in DKO cells.

Significant to the self-renewal properties of ES cells, we detected reduction in the expression levels of pluripotent factors. *Nanog* was down regulated 1.81-fold on the array and 4.6-fold by qRT-PCR. In addition, *Oct4* (*Pou5f1*), *Rex1* (*Zfp42*), *Essrb*, and *Zfx*, were all significantly reduced between 1.45- and 1.63-fold (Figure 4.10C). We further performed Western blots on protein extracts from control and day3 DKO cells and found that the protein levels of Oct4 and Nanog were reduced in parallel with the decrease of HDAC1/2 activity (Figure 4.12 A, B). We analyzed an additional 39 genes (80 probes) associated with pluripotency and observed a progressive loss of pluripotent factor expression over 3-day time period in which HDAC1/2 activity is lost ($R^2 = 0.96$; $p = 0.019$). However, analysis of 111 genes associated with stem cell differentiation showed only a weak positive correlation which was not significant ($R^2 = 0.66$; $p = 0.186$) (Figure4.12 C and appendix table 3).

These results suggest that HDAC1/2 are required for the expression of pluripotent factors, Nanog and Oct4, which explained the change in cell morphology of DKO cells at day3 (Figure 4.3B). However, loss of HDAC1/2 is not sufficient to depress genes associated with early differentiation.

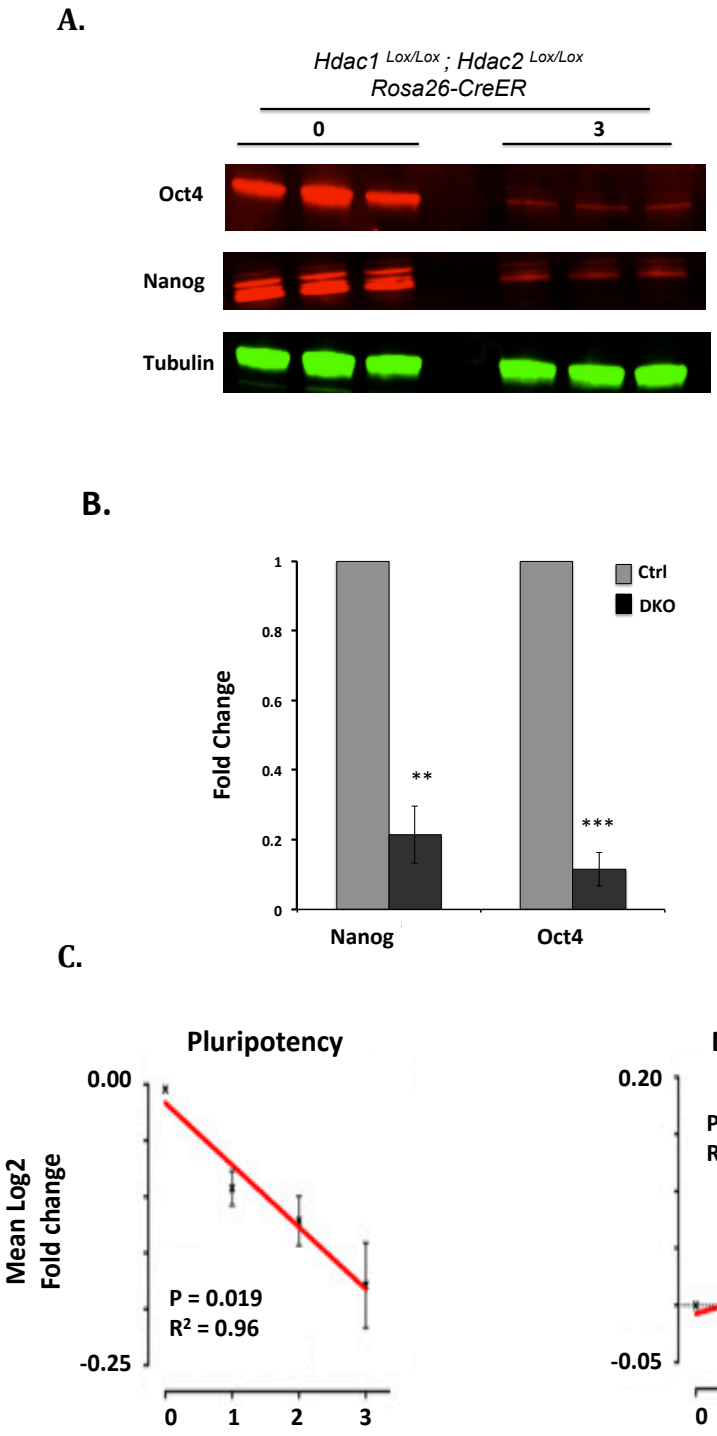


Figure 4.12: HDAC1/2 are required for the expression of Oct4 and Nanog. (A) Quantitative Western blot data for Oct4 and Nanog proteins indicate reduction in parallel with the decrease of HDAC1/2 activity. (B) Quantitative western blot data for the indicated proteins were performed using an Odyssey scanner and normalized to the level of α-tubulin. (C) Regression analysis of pluripotency and differentiation associated genes using mean log2 fold changes of microarray data. Significance (*P* value) was calculated using a two-tailed t test (***P* < 0.001, ****P* < 0.0001).

4.2.8 HDAC1 and an HDAC1-2 chimera are able to rescue DKO ES cells viability

It seemed likely that the essential requirement for HDAC1/2 in cell division and their ability to influence gene expression was dependent upon deacetylase activity. To confirm this, DKO cells were transfected with cDNAs for HDAC1 wild type and catalytically inactive version of HDAC1 ($HDAC1^{Y303H}$) to examine their ability to rescue the cell viability. The catalytically inactive $HDAC1^{Y303H}$ was generated by a site directed mutation of Tyrosine303 to Histidine that reduced HDAC1 enzymatic activity (Fischle, W., et al. 2002).

cDNAs were generated by PCR and sub-cloned into a pCAG-IRES-eGFP plasmid using In-Fusion HD EcoDry Cloning Plus kits (Clontech). The eGFP (enhanced green fluorescent protein) tag was used to assess the transfection efficiency as well as select eGFP-positive cells by using fluorescence-activated cell sorting (FACS).

ES cells were transfected with 5 μg of wt-HDAC1 and $HDAC1^{Y303H}$, and cultured for 48hours before sorting for GFP-positive (transfected) cells using a FACS. The FACS analysis showed the percentage of ES cells expressing HDAC1 constructs (between 42% to 45%) (Figure 4.13), and western blots with anti-Flag antisera revealed the level of wild-type and chimeric HDAC1 expression (Figure 4.14B). Transfected cells (FACS sorted for GFP expression) were then treated with 4- hydroxytamoxifen (OHT) (for 24 hours) and cultured for a further 4 days before cell counting. As seen in figure 4.13C, wild-type HDAC1 was able to rescue DKO cells, while the majority of cells transfected with $HDAC1^{Y303H}$ died at day 4 following *Hdac1/2* inactivation.

This result prompted us to use the DKO cells as a model system in which to interrogate aspects of HDAC1 activity. HDAC1, 2, and 3 share a highly conserved catalytic domain and a divergent C-terminal domain that is subject to post-translation modifications which are thought to regulate protein stability, catalytic activity and complex formation (Hassig, C.A. et al., 1997). We therefore swapped the C-terminal domain between HDAC1and HDAC2 (HDAC1-2 CTT); and HDAC1 with HDAC3 (HDAC1-3 CTT). As observed in figure 4.14C, HDAC1-2 CTT was able to rescue cell viability, while most of cells transfected with HDAC1-3CTT died at day 4 following *Hdac1/2* deletion. However, the expression levels of HDAC1-3 CTT was very low compared to other constructs (Figure 4.14C). This result suggests that, C- terminal tails of HDAC1 and HDAC2 are essential for their catalytic activity and the C-terminal tail of HDAC3 is less efficient.

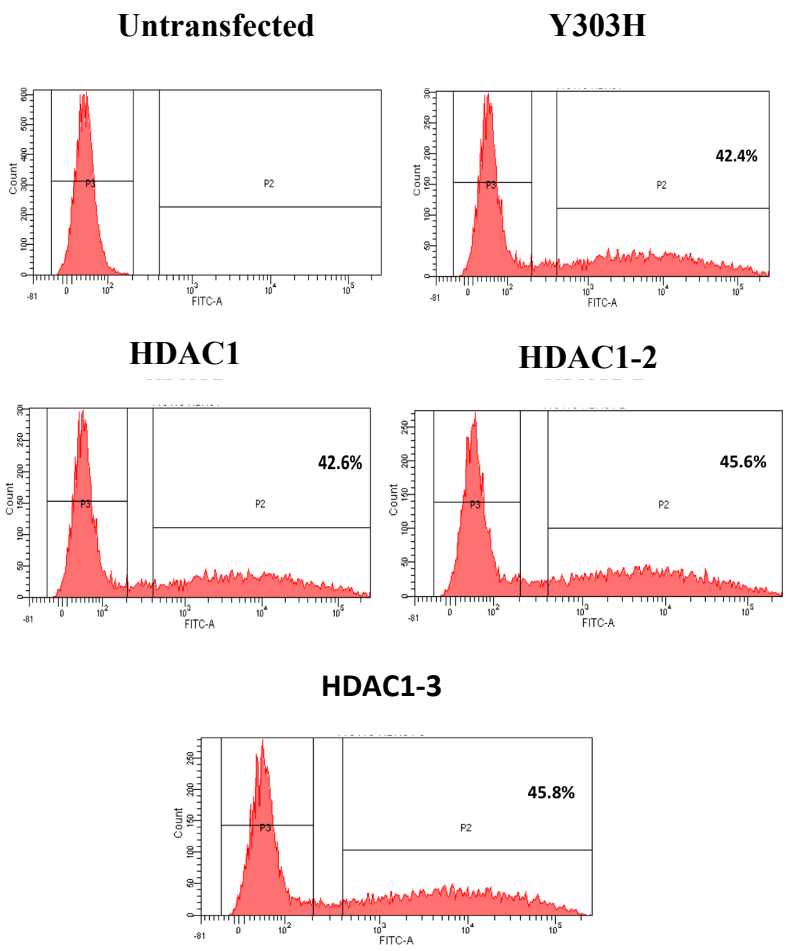
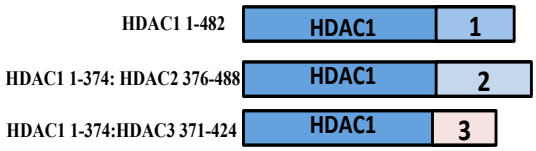


Figure 4.13: Transfection efficiency of HDAC1 constructs 48 hours after transfection. FACS analyses identify percentages of GFP-positive transfected cells for HDAC1, HDAC1^{Y303H}, HDAC1-2, HDAC1-3 constructs and untransfected cells used as control. Transfection efficiency between 42-45% for each construct.

A.



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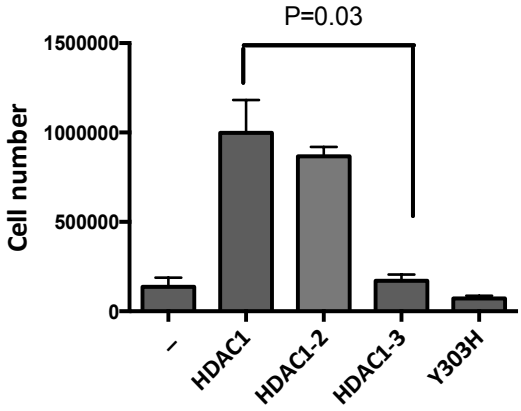


C.

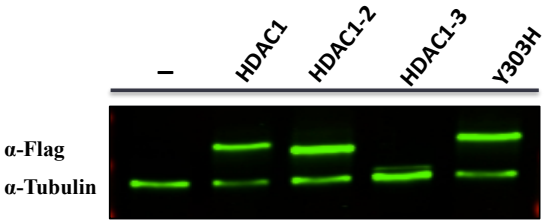
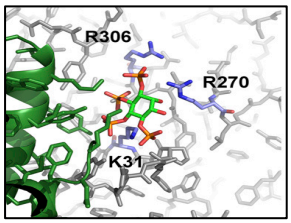


Figure 4.14: HDAC1 and HDAC1-2 chimera are able to rescue DKO ES cells viability. (A) Schematic diagram of the HDAC1 mutant constructs. (B) The number of viable cells, transfected with the indicated HDAC1 expression constructs, were counted 4 days after *Hdac1/2* inactivation. (C) Western blot performed with anti-FLAG and anti-α-tubulin antisera to determine the relative expression level of individual HDAC1 constructs. All values are mean (n>3) ± SEM. Significance (*P* value) was calculated using a two-tailed t test.

4.2.9 Rescue of DKO cells is dependent upon the integrity of HDAC1 IP_4 binding pocket

It has been recently shown that the activity of HDAC1 and HDAC3 is modulated by Inositol tetraphosphate (1,4,5,6) P4, (IP4) (Millard, C., et al 2013, Watson, P., et al. 2012). As shown in figure 4.14A, The IP4 binding pocket on the surface of HDAC1 is made up of a number of positively charged residues (K31, R270, and R306), which form hydrogen bonds with the negatively charged phosphate of IP_4. To test the requirement for IP4 binding to the activity of HDAC1. DKO cells were transfected with 5 forms of mutated HDAC1, lysine 31, arginine 270, and arginine 306 were mutated to glutamine, a polar non-charged residue, individually (K31Q, R270Q, R306Q), as double mutants (K31Q/R270Q, R270Q/R306Q), or as a triple mutants (K31Q/R270Q/ R306Q). As shown in figure (4.15B), the deacetylase activity of individual mutants was reduced, the double mutants showed a lower activity compared to the individual and the triple mutants had lowest deacetylase activity of all.

A.



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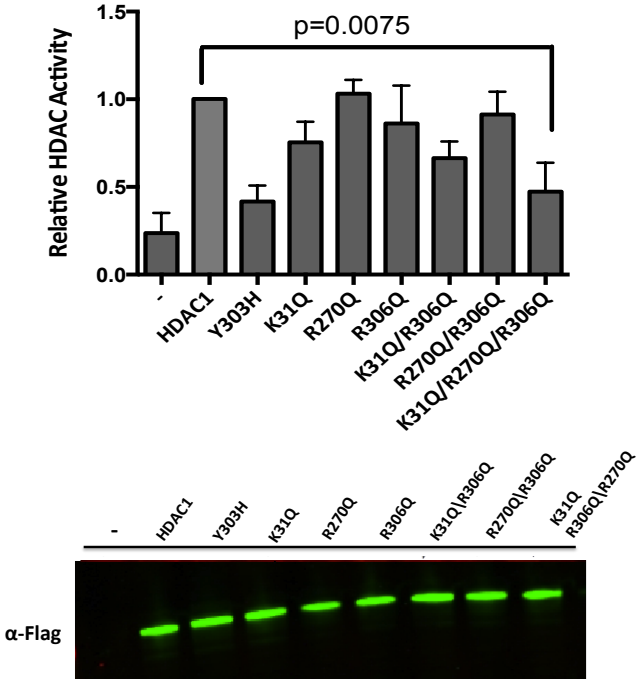


Figure 4.15: Catalytic activity of HDAC1 is dependent upon the integrity of the inositol tetraphosphate (IP_4) binding pocket. (A) Structure of HDAC1deacetylase domain with positively charged residues critical for the interaction with IP_4 (K31, R270, and R306) marked in blue. (B) Relative deacetylase activity was measured using individual Flag-tagged HDAC1 constructs immunoprecipitation using anti-Flag antisera. All values are means (n>3) ± SEM and are normalised relative to the level of protein expression (Shown in the lower panel). Significance (*P* value) was calculated using a two-tailed t test. Western blot performed with anti-FLAG and anti-α-tubulin antisera to determine the relative expression level of individual HDAC1 constructs.

We further tested the ability of the mutant HDAC1 constructs to rescue the viability of the DKO cells at 4 days following gene inactivation (Figure 4.16). We also observed that, the single-point mutations (K31Q, R270Q, R306Q) produced a lower number of viable cells compared with controls, whereas double and triple mutation resulted in an additive effect, with the smallest number of viable cells. These results indicate that, IP4 binding is necessary for the full activity of HDAC1 in vivo.

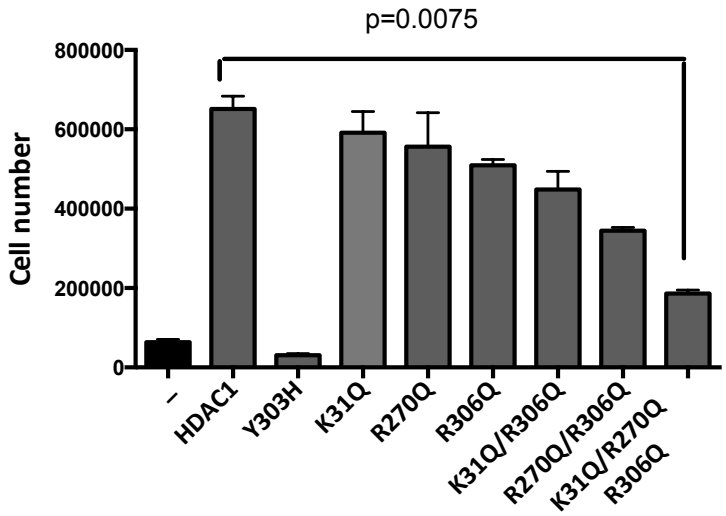


Figure 4.16: Cell viability is dependent upon the integrity of the inositol tetraphosphate (IP_4) binding pocket. The number of viable cells, transfected with the indicated HDAC1 expression constructs, were counted 4 d after *Hdac1/2* inactivation. All values are means (n>3) ± SEM. Significance (*P* value) was calculated using a two-tailed t test.

We then wanted to test the ability of HDAC1 mutants to bind their binding partners (Sin3A and MTA2) in co-repressor complexes. To do this, HDAC1 mutants were co-immunoprecipitated with endogenous Sin3A and MTA2. As shown in figure 4.17, Sin3A and MTA2 were immunoprecipitated from all single-point mutation except K31Q mutant. However, double and triple mutants were unable to immunoprecipitate the endogenous proteins. Together these results suggest that the IP4 pocket may also essential for binding of HDAC1 to their protein partners and formation of co-repressor complexes.

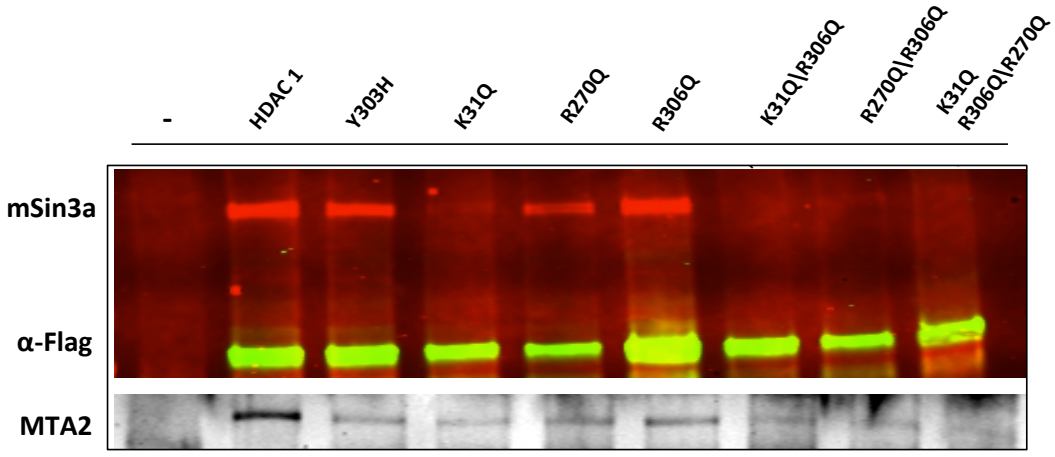


Figure 4.17: The IP4 pocket may also be essential for binding of HDAC1. Flag-tagged HDAC1 constructs immunoprecipitation using anti-Flag antisera followed by anti-Sin3A and anti-MTA2 western blot to detect pulled down endogenous proteins (Sin3A and MTA2).

4.3 Conclusions

We have generated ES cells in which *Hdac1* and *Hdac2* can be simultaneously inactivated. Loss of HDAC1/2 results in a 60% reduction in HDAC activity (figure 4.2) as well as loss of cell viability 4 days after gene inactivation (figure 4.3A). The lethal phenotype is dependent on an active cell cycle by stimulating cell cycle exit before inactivation of *Hdac1/2* by using retinoic acid (RA) or generating embryoid bodies (EBs), the majority of cells remained viable (Figure 4.5). DKO cells at day3 show a significant increase in DNA abnormalities and segregation defects that is likely the major cause of cell death.

Analysis of global histone acetylation reveals a relatively modest increase in the acetylation levels at most sites that analyzed which is consistent with the reduction in the integrity of co-repressor complexes in the absence of both enzymes (Figure 4.8).

Loss of HDAC1/2 activity correlates with the down-regulation of almost 2,000 genes including the pluripotent factors, which suggests that HDAC1/2 are required for the expression of Oct4 and Nanog (Figure 4.12). A recent study revealed the binding of HDAC1 close to the transcription start site of pluripotent factors, including Oct4, Nanog, Sox2, and Rex1, suggesting a positive role in the maintenance of cell self-renewal (Kidder et al. 2012)

Recently, it was shown that, using in vitro assays, activity of HDAC1 and HDAC3 is modulated through the binding of IP4. Mutations of residues that abolish IP4 binding reduce HDAC1 activity in vivo (Figure 4.15).

Chapter 5: Understanding the role of HDAC1 and HDAC2 in ES cells differentiation

5.1 Chapter aims

Using *Hdac1*$^{Lox/Lox}$, *Hdac2*$^{Lox/WT}$ ES cells (Chapter 3) I aimed to further elucidate the role of HDAC1/2 in the differentiation of ES cells. The differentiation of *Hdac1*^{KO}; *Hdac2*^{Het} cells was assessed using two different differentiation assays, generating embryoid bodies (EBs), and using LIF-free /Serum-free medium.

Further, I aimed to identify genes directly regulated by HDAC1/2, by treatment of ES cells with retinoic acid (RA) which is effective way of activating a well characterized gene expression programme. Approximately 200 genes are induced within 6 hours, in particular, HOX genes, which are retinoic acid (RA) primary response genes. The induction of retinoic acid (RA) target genes was compared between control (untreated) and *Hdac1*/2 deleted cells (DKO) (chapter 4).

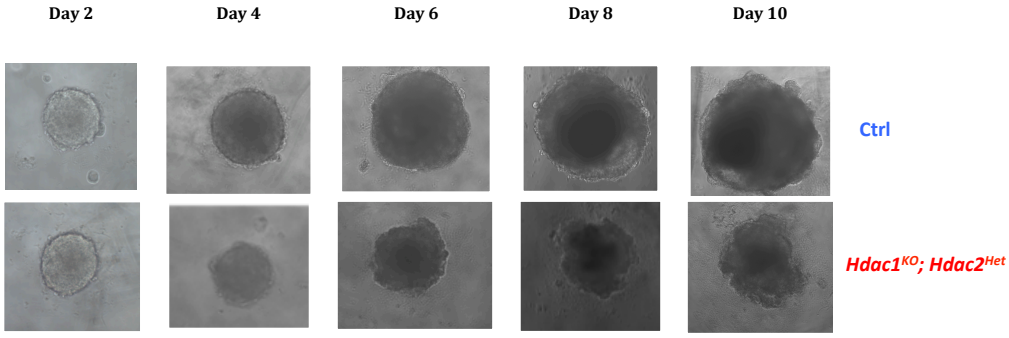
5.2 Results

5.2.1 Increased expression of cardiomyocyte markers in *Hdac1^{KO}; Hdac2^{Het}* embryoid bodies (EBs)

It has been previously shown that, loss of HDAC1 causes enhanced EBs differentiation into mesoderm and ectoderm cell linages (Dovey, O.M. et al, 2010). Embryoid bodies lacking HDAC1 were reduced in size and exhibited induction of the cardiomyocyte specific markers. In chapter 3 we found that, the differentiation ability of *Hdac1^{KO}; Hdac2^{Het}* ES cells (KO) was not inhibited, cells were able to differentiate upon LIF withdrawal (Figure 3.7). Therefore, further differentiation analyses were required to investigate the role of HDAC1 and HDAC2 in greater detail.

Hdac1^{KO}, Hdac2^{Het} ES cells were used to generate embryoid bodies (EBs). Control (untreated) and KO (OHT- treated) cells were cultured in the absence of LIF and plated onto Corning® Costar® Ultra-Low attachment plates which prevented cell adhesion and produced a uniform size of EBs similar to the hanging drop method. EBs were cultured for 10 days and visualized every 2 days. As shown in figure 5.1A, control and *Hdac1^{KO}; Hdac2^{Het}* cells were able to aggregate and form EBs over a two-day period. However, extended culture revealed that EBs derived from *Hdac1^{KO}; Hdac2^{Het}* cells were irregular and reduced in size compared to control (Figure 5.1 A and B). The reduction in size implied increased differentiation of EBs derived from *Hdac1^{KO}; Hdac2^{Het}* compared to controls.

A.



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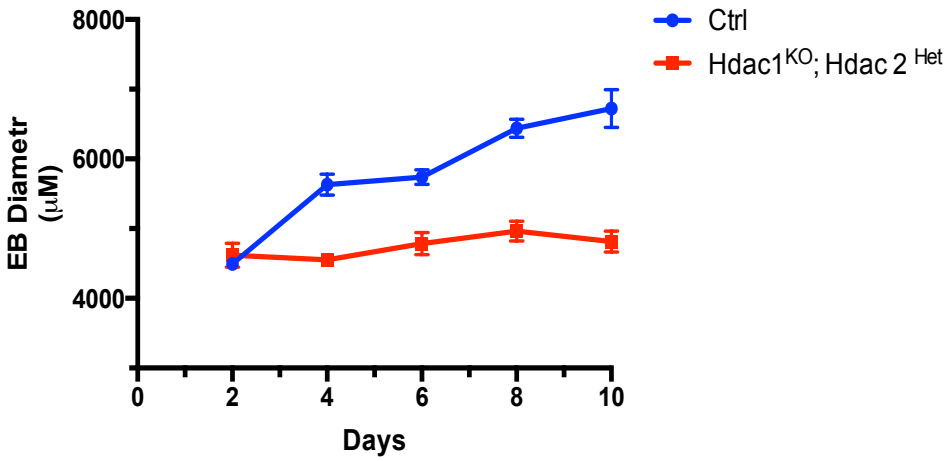


Figure 5.1: Loss of HDAC1/2 effects embryoid body differentiation (EBs). (A) Images representative of EBs at indicted time points shows that *Hdac1*^{KO}*; Hdac2*^{Het} EBs (OHT treated) are reduced in size. (B) Mean size of EBs during a 10-days experiment. All values are mean (n = 3) ±SEM.

Gene expression analysis of EBs was performed to determine cell types present during EB differentiation. Quantitative RT-PCR was performed for lineage specific markers using mRNA isolated from control and *Hdac1^{KO}; Hdac2^{Het}* EBs over a 10-day period. As observed in figure 5.2, HDAC1^{ko}; HDAC2^{Het} EBs were able to repress *Nanog* (a stem cell marker), indicating that KO cells are able to exit pluripotent state. As demonstrated previously (Dovey, O.M. et al, 2010), EBs lacking HDAC1 were differentiated toward mesodermal lineage, therefore we examined the expression patterns of cardiomyocyte-specific markers. *NKX2-5* and *Mef2c* are transcription factors essential for differentiated cardiomyocyte and heart development. Induction of *NKX2-5* and *Mef2c* expression was observed in EBs derived from control and *Hdac1^{KO}; Hdac2^{Het}* by day4 when *Nanog* has already been repressed (Figure 5.2). However, transcript level of both genes was higher in EBs derived from *HDAC1^{ko}; HDAC2^{Het}* than that in control. Additionally, *TBX5* (an early cardiomyocyte marker) and *TNNT2* (a late cardiomyocyte marker) were induced in a similar pattern, with a higher expression in EBs derived from *HDAC1^{ko}; HDAC2^{Het}* compared to control. *TBX5* was induced by day 4 in control EBs, earlier than EBs derived from *Hdac1^{KO}; Hdac2^{Het}*. Notably, the induction of *TNNT2* occurred at day 6 later than other cardiomyocyte markers, and again the transcript level was higher in EBs derived from *Hdac1^{KO}; Hdac2^{Het}* compared to controls (Figure 5.2). Together, these result suggest that the differentiation into cardiomyocyte (mesodermal) lineage is enhanced in cells with reduced levels of HDAC activity.

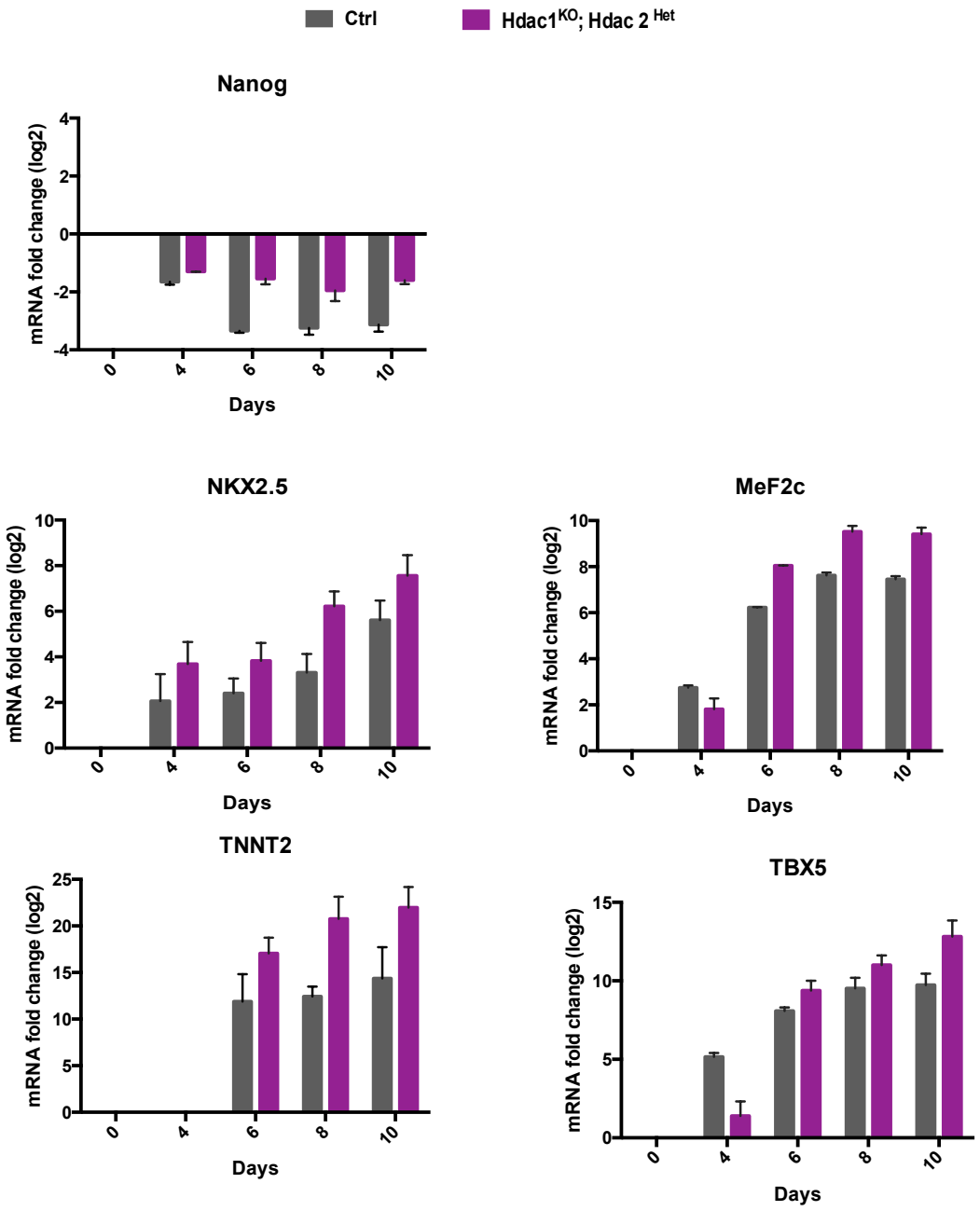


Figure 5.2: Loss of HDAC1/2 enhanced cardiomyocyte differentiation of EBs. Quantitative RT-PCR data of undifferentiated stem cell marker (*Nanog*), cardiomyocyte specific markers (*NKX2-5*, *Mef2c*), early cardiomyocyte marker (*TBX5*), and late cardiomyocyte marker (*TNNT2*) was performed on mRNA collected from control (untreated) and $Hdac1^{KO}$; $Hdac2^{Het}$ (OHT treated) at days 0,4,6,8, and 10 days during EB differentiation. All values are mean (n = 3) ±SEM. Values indicate expression of gene relative to the *Gapdh* reference gene, measured using Universal ProbeLibrary hydrolysis probes.

We then examined the expression level of *MyoD*, a skeletal muscle marker, and *GATA4*, an endoderm linage marker. As shown in figure 5.3, *MyoD* showed a similar induction through the same period, levels were higher in EBs derived from $Hdac1^{KO}$; $Hdac2^{Het}$ compared to control, after 10 days of differentiation it exhibited a significant increase with a 8-fold in $Hdac1^{KO}$; $Hdac2^{Het}$ EBs compared to 5.6-fold in EBs derived from control. This result further confirmed that, induced differentiation toward mesodermal linages in EBs lacking HDAC1 and with 50% of HDAC2. However, endoderm marker *GATA4* did not show a similar induction, we detected only a slight increase in the expression level of *GATA4* in $Hdac1^{KO}$; $Hdac2^{Het}$ EBs compared to controls (Figure 5.3).

Altogether, these results indicate that induced differentiation of $HDAC1^{KO}$; $HDAC2^{Het}$ ES cells, via formation of EBs in LIF-free media, resulted in increased expression of cardiomyocyte-specific markers, which reveal their role in control cardiac differentiation.

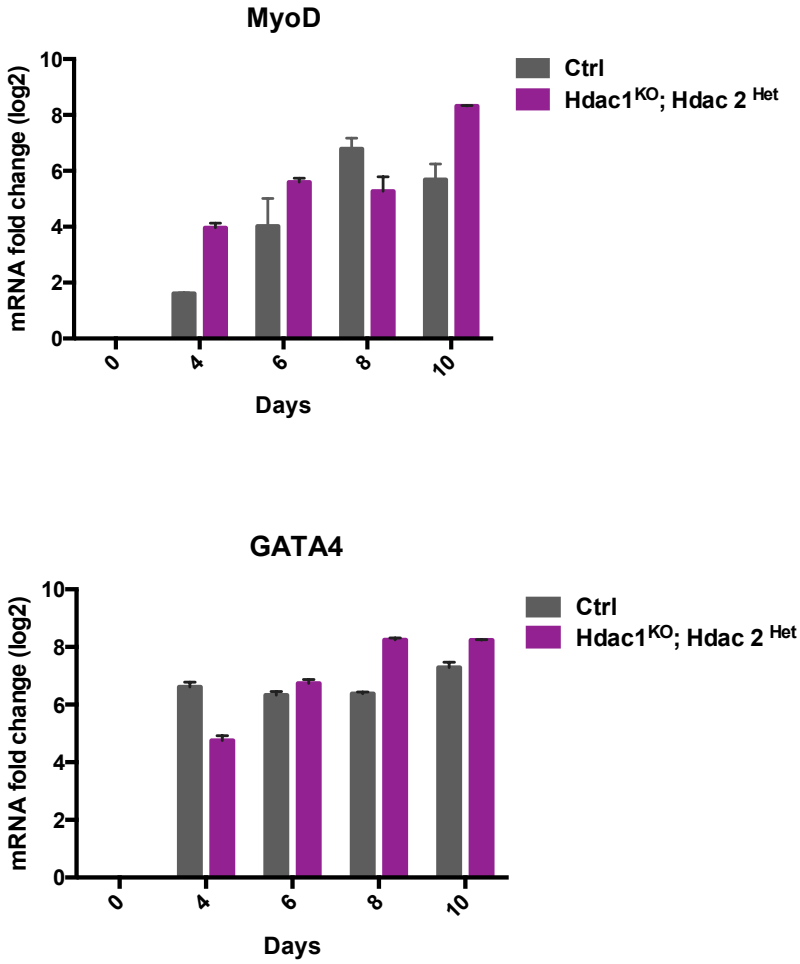


Figure 5.3: Differentiation of ES cells lacking HDAC1/2 is associated with a slight induction of mesoderm and endoderm markers. Quantitative RT-PCR data for skeletal muscle mesoderm (*MyoD*) and endoderm (*GATA4*) marker was performed on mRNA collected from control (untreated) and *Hdac1*^{KO}*; Hdac2*^{Het} (OHT treated) at days 0, 4, 6, 8, and 10 days during EB differentiation. All values are mean (n = 3) ±SEM. Values indicate expression of gene relative to the *Gapdh* reference gene, measured using Universal ProbeLibrary hydrolysis probes.

5.2.2 Differentiation of *Hdac*1^{KO}; *Hdac2*^{Het} ES in serum-free (N2B27) media

Maintenance of the undifferentiated state of mES cells in vitro requires addition of LIF to the growth media. Leukaemia inhibitory factor (LIF) acts by activating the STAT3 signaling pathway that helps maintain pluripotency by blocking endodermal and mesodermal differentiation. However, it has been shown that LIF is insufficient to block neuronal differentiation in serum-free mES cell culture medium, therefore, the presence of serum is required. Bone morphogenic protein (BMP4) was identified as the constituent of serum, signaling through SMAD proteins, which promotes expression of Inhibitor-of-differentiation (Id) proteins that suppress ectodermal differentiation (Ying, Q., et al. 2003). Accordingly, LIF and serum (BMP4) are required to maintain pluripotency and suppress differentiation of mES cells in vitro. Therefore, another differentiation assay was performed in N2B27 media (lacking LIF and serum (BMP4)) to examine the effect of deletion HDAC1^{KO}; HDAC2^{Het} on ES cell differentiation under serum-free conditions

Control (untreated) and HDAC1^{KO}; HDAC2^{Het} (KO) (OHT treated) ES cells were cultured in LIF-free, serum-free (N2B27) media for 6 days culture period. ES cells were plated onto laminin-coated dishes to enhance cell viability and induce neuronal differentiation. Removal of LIF releases the inhibitory effects of STAT3 on mesoderm and endoderm differentiation while removal of BMP prevents inhibitory effects of Id on neuroectoderm differentiation.

In order to identify cell types present during differentiation, mRNA was collected from control (untreated) and *Hdac1^{KO}; Hdac2^{Het}* (OHT treated) cells at 0, 2, 4, and 6

days and quantitative RT-PCR was performed for linage specific markers. The undifferentiated (stem cell) marker, *Nanog*, was used as a control and was found to be repressed in control and $Hdac1^{KO}; Hdac2^{Het}$ cells indicating that these cells exit the pluripotent program when cultured in LIF-free and serum-free culture media (Figure 5.4)

Differentiation of mES cells using serum-free media induced neuroectoderm lineage, which gives rise to neurons. The neuroectoderm lineage is characterized by the expression of transcription factor *SOX1* (Suter, D.M., et al 2009). SRY-Related HMG-Box Gene 1 (SOX1) plays a role in development and maintenance of neuroectodermal stage. The quantitative RT-PCR data indicated that the induction of neuroectoderm lineage was unaffected in $Hdac1^{KO}; Hdac2^{Het}$ cells, since there was no consistent difference in the activation of *SOX1* compared to control cells (Figure 5.4). A 3-fold induction was observed in control compared to 2-fold in $Hdac1^{KO}; Hdac2^{Het}$ cells at day 2, however at day 4 it increased in $Hdac1^{KO}; Hdac2^{Het}$ while stayed the same in control (4.5-fold compared to 3-fold). To further confirm that we had induced differentiation of neuronal lineage we also examined expression of the transcription factor *PAX6* (Figure 5.4). *PAX6* is activated later than *SOX1*, (1.07- compared to 3.4-fold at day 2), which is expected as neurons further differentiated toward radial glia they switch from *SOX1* to *PAX6* expression (Suter, D.M., et al 2009). We noticed that the expression level of both *SOX1* and *PAX6*, are not significantly changed in $Hdac1^{KO}; Hdac2^{Het}$ cells compared to controls, which suggests that the differentiation is unaffected (Figure 5.4).

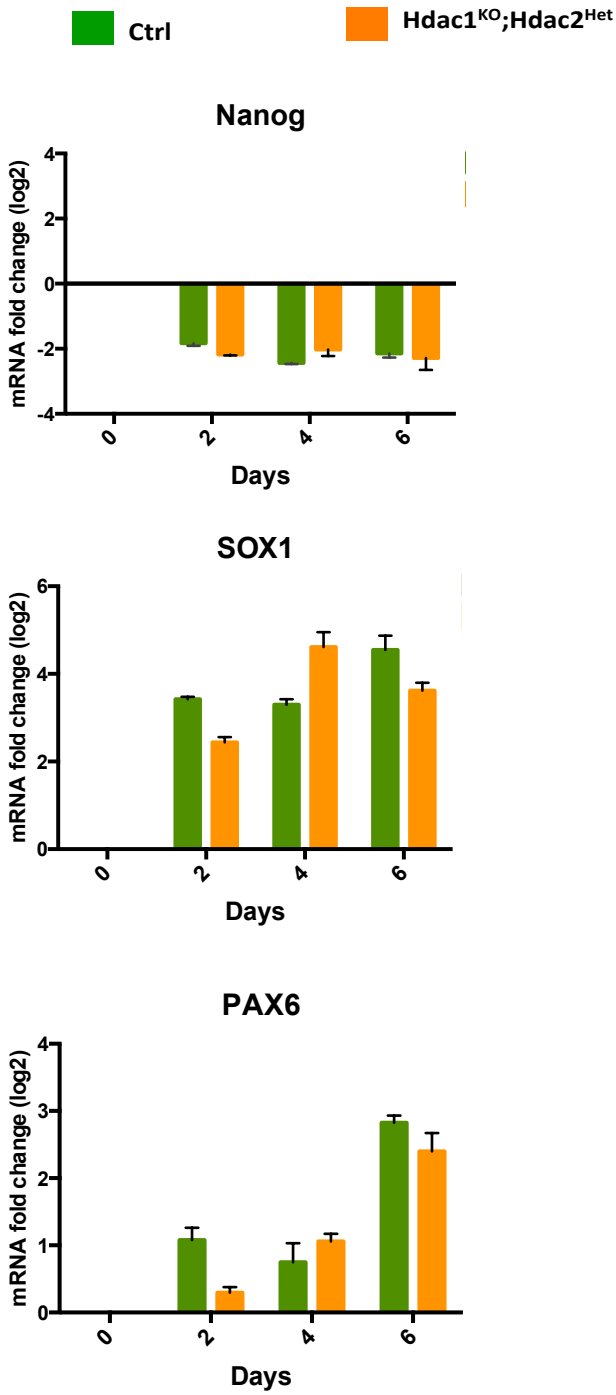


Figure 5.4: Differentiation of *Hdac1*^{KO}, *Hdac2*^{Het} ES cells in serum-free media. Quantitative RT-PCR data for undifferentiated stem cell marker (*Nanog*), and neuroectodem transcription factors (*SOX1* and *PAX6*) was performed on mRNA collected from control (untreated) and *Hdac1*^{KO}; *Hdac2*^{Het} (OHT treated) cells cultured in serum-fee (N2B27) media on days 0, 2, 4, and 6. All values are mean (n=3) ±SEM. Values indicate expression of gene relative to the *Gapdh* reference gene, measured using Universal ProbeLibrary hydrolysis probes.

We further examined the expression levels of neuronal specific markers. The expression level of *ASCL1*, a transcription factor that plays an essential role in neuronal commitment and differentiation, was up-regulated at day 2 and there was no significant difference in the expression between control and $Hdac1^{KO}; Hdac2^{Het}$ (1.8- compared to 1.3-fold) (Figure 5.5). Control and $Hdac1^{KO}; Hdac2^{Het}$ cells showed a similar induction of *CXCR4*, which plays a role in neuronal guidance, and *FZD9* that is expressed in neural precursor cells (Figure 5.5). However, the expression level of *CXCR4* and *FZD9* were slightly decreased by day6 in $Hdac1^{KO}; Hdac2^{Het}$ (4.7- and 4.2-fold) compared to control (4.8- and 5.7-fold change). Additionally, *Noggin* (expressed in neural precursor cells) was induced in a similar pattern at day 2, with a small difference in the expression level between control and $Hdac1^{KO}; Hdac2^{Het}$ cells (2.5-fold compared to 3.7-fold), then it increased to 9.4-fold in control and 8.6-fold in $Hdac1^{KO}; Hdac2^{Het}$ (Figure 5.5). Overall, These results suggest that under these differentiation conditions, ES cells lacking HDAC1/2 are able to differentiate toward neuroectoderm linage, and there is no significant change in the expression of neuroectoderm markers compared to control cells.

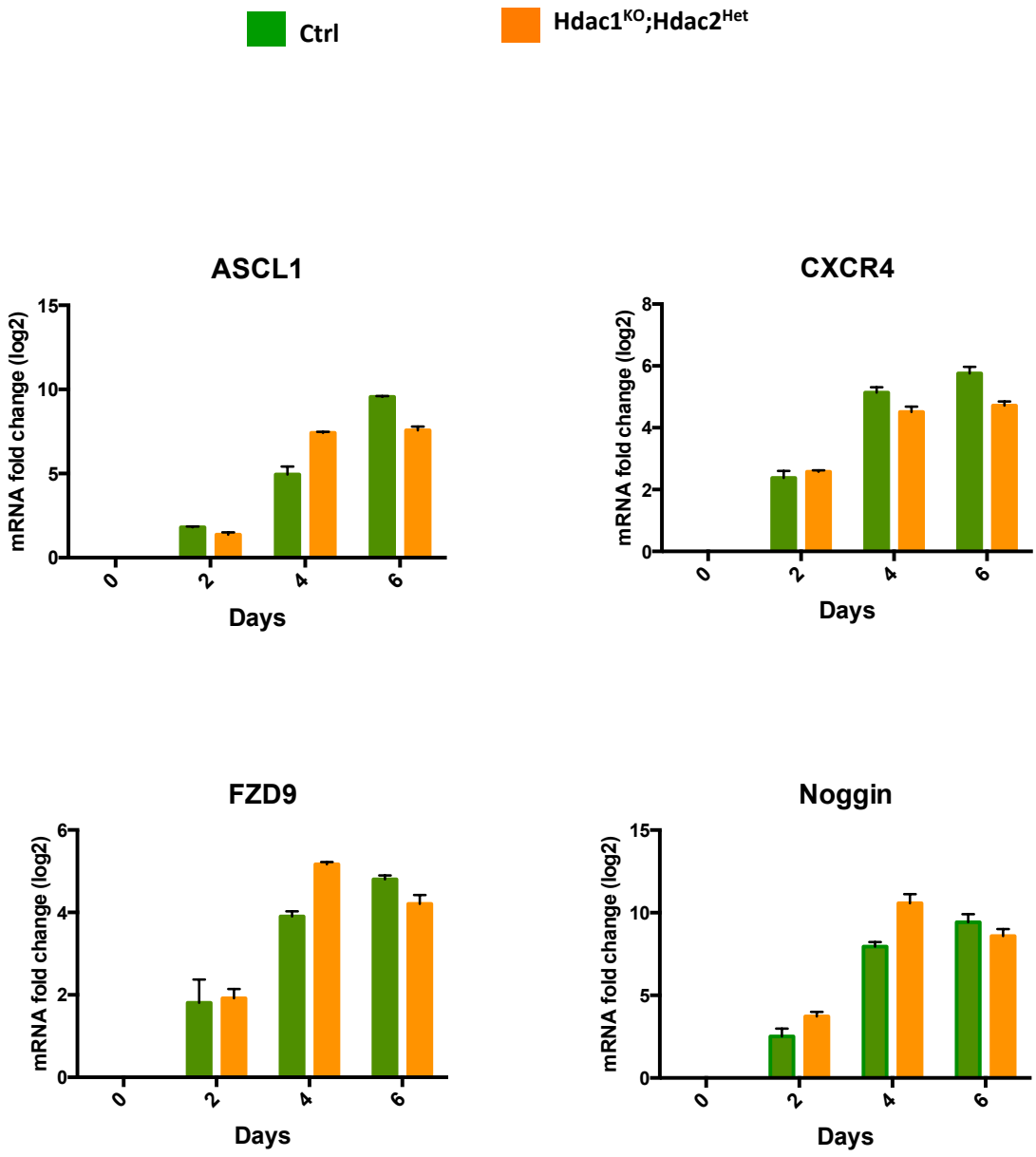


Figure 5.5 Expression of neuronal specific markers in *Hdac1^{KO}; Hdac2^{Het}* ES cells. Quantitative RT-PCR data for neuronal specific markers: *ASCL1, CXCR4, FZD9*, and *Noggin*, was performed on mRNA collected from control (untreated) and *Hdac1^{KO}; Hdac2^{Het}* (OHT treated) cells cultured in serum-fee (N2B27) media on days 0, 2, 4, and 6. All values are mean (n=3) ±SEM. Values indicate expression of gene relative to the *Gapdh* reference gene, measured using Universal ProbeLibrary hydrolysis probes.

5.2.3 HDAC1/2 positively regulate expression of HOX genes following RA treatment

As previously demonstrated in chapter 4, the transcriptome analysis of *Hdac1/2* deleted ES cells revealed that approximately 2000 genes are deregulated. Furthermore, there is correlation between the reduction in HDAC activity and the number of deregulated genes (Figure 4.9), many genes were down-regulated as well as up-regulated which suggests that HDAC1/2 may also play a positive role in the expression of some genes. We also found that the expression levels of *Oct4* and *Nanog* were down-regulated when HDAC1/2 are lost in ES cells (Figure 4.11). To identify direct effects of HDAC1/2 on gene expression, we compared the induction of retinoic acid (RA) target genes between control (untreated) and *Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/Lox}$; CreER (**DKO)** ES cells (OHT treated). All-trans-retinoic acid (RA) is a metabolic product of vitamin A that plays a role in regulating ES cell differentiation.

Cells were cultured in the presence of 4-hydroxytamoxifen (OHT) for 24 hours to induce the deletion of *Hdac1/2*, and then 2.5-days following gene inactivation (at point which both proteins are lost), cells were then treated with 1μM retinoic acid (RA) for 6 hours. mRNA was isolated from control, and DKO with , and without RA to perform a comparative microarray analysis. Transcripts up-regulated or down-regulated by ≥1.4-fold change (FC ≥1.4, adjusted $P<0.05$) were identified from three independent experiments using ArrayTrack analysis software (appendix table 6). Adding RA resulted in a change in the expression of 238 transcripts (C versus C+RA), with 167 up-regulated and 71 down-regulated transcripts (Figure 5.6). A total of 195 deregulated

transcripts were detected in DKO+RA compared to DKO as a result of adding RA, of these, 115 transcripts were up-regulated and 79 down-regulated.

Analysis of the expression level of pluripotent factors revealed that, *Nanog* and *Oct4 (Pou5f1),* were uniformly expressed (unchanged) in all samples treated with RA (Figure 5.7A). However, transcript levels of pluripotent factors were significantly reduced in DKO compared to control (Figure 5.7B), in agreement with our previous result (chapter 4) demonstrating that HDAC1/2 regulate expression of pluripotent factors.

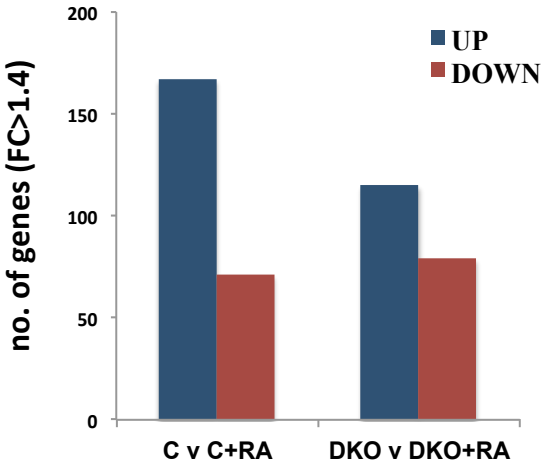
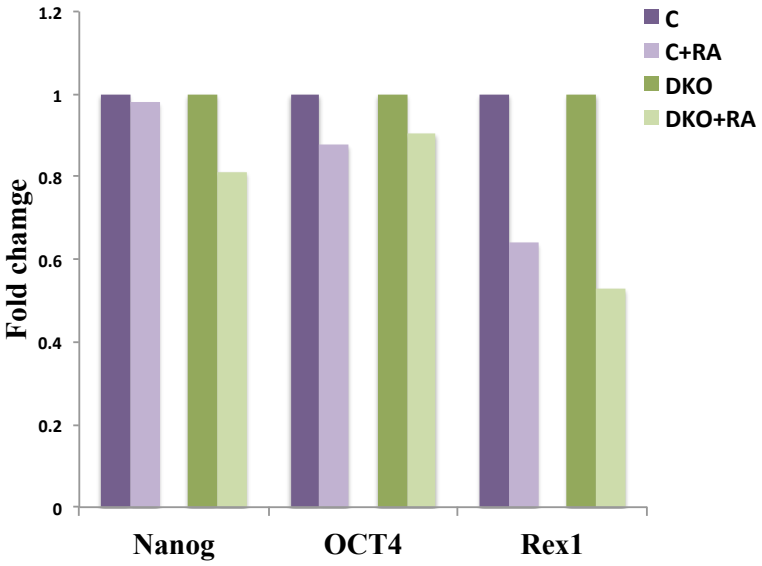


Figure 5.6: Number of differentially expressed genes in RA treated cells. Control and DKO (*Hdac*1$^{Lox/Lox}$; *Hdac*2$^{Lox/Lox)}$ cells were cultured with OHT for 24h, and then 2.5-day later were treated with 1μM retinoic acid (RA) for 6 hours. A comparative microarray analysis was performed on mRNA isolated from C, C+RA, DKO, and DKO+RA. Transcripts deregulated ≥1.4 ($P<0.05$) were identified from three independent experiments.

A.



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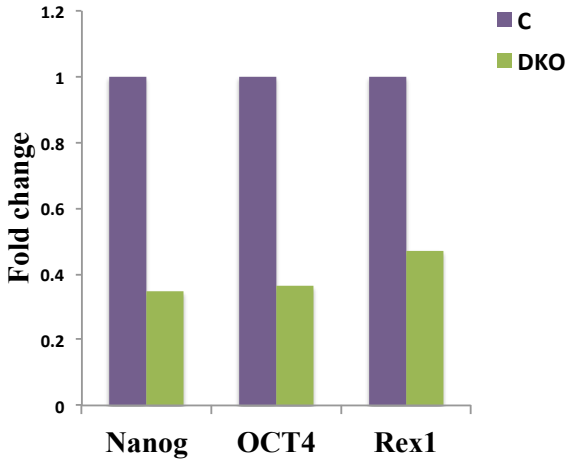


Figure 5.7: Expression levels of pluripotent factors are unchanged at six hours following RA treatment. (A) Expression of *Nanog*, *Oct4* (*pou5f1)* and *Rex1* (*Zfp42)* were compared between control treated with RA(C+RA) versus control and between DKO treated with RA (DKO+RA) versus DKO, (B) and between control versus DKO cells. Fold change was calculated using microarray data.

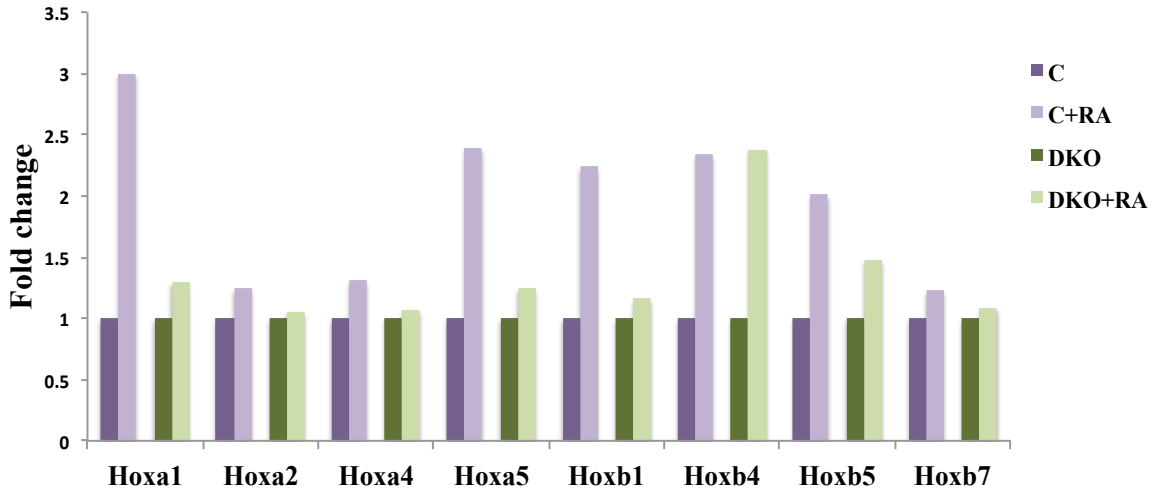
RA acts by binding to the retinoic acid receptor RARs (RARα, RARβ, and RARγ), a member of the nuclear receptor family, in combination with retinoic X receptors RXRs (RXRα, RXRβ, and RXRγ). In the nucleus, RA-bound RAR/RXR dimers induce expression of target genes by binding to DNA sequences known as retinoic acid response elements (RAREs) (Rochette-Egly, C., et al, 2009). Addition of RA induced transcription of primary RA response genes that possess RAREs in proximity to the transcription sites e.g. HOX genes, within two hours of treatment.

Therefore, we first analyzed the expression pattern of HOX genes (direct targets of RA) between RA treated and untreated cells. We found that six hours following RA treatment, transcript levels of HOX genes (Hoxa1, Hoxa5, Hoxb1, etc.) were significantly up-regulated (between 3-fold and 2-fold change) in control treated with RA (C+RA) compared to untreated control (C) (Figure 5.8A), demonstrating that RA is efficiently inducing their expression. Interestingly, DKO cells treated with RA (DKO+RA) showed reduced induction through the same time period. The transcript levels of almost all HOX genes were unchanged in DKO+RA compared to DKO cells (Figure 5.8A). Notably, the expression level of Hoxa1 was significantly induced by 2.9-fold in C+RA, and only 1.2-fold in DKO+RA. Furthermore, the transcript levels of the Hoxa5 and Hoxb1 were also induced between 2.3- and 2.2-fold on C+RA, while in the DKO+RA they induced between 1.2- and 1.1-fold (Figure 5.8A), suggesting a positive role of HDAC1/2 in their induction.

However, HOXb4 was induced to the same level in RA treated cells (C+RA) and (DKO+RA) by 2.3-fold change. Moreover, HOXb5 was increased modestly (1.4-fold) in DKO+RA compared to 2-fold in C+RA following RA treatment (Figure 5.8A).

The lack of change in HOX genes expression observed in DKO+RA cells prompted us to examine the transcription levels of more RA primary response genes. *CYP26a* (cytochrome P450 26 subfamily) that mediates RA catabolism (Pennimpede T. et al., 2010) was significantly induced by 13-fold in C+RA, but only 9.7-fold in DKO+RA (Figure 5.8B). Furthermore, addition of RA resulted in reduced induction of the transcript levels of *Cdx1* (Caudal Type Homeo-Box Transcription Factor 1), *Meis2* (Homeobox protein Meis2), and *Stra8* (stimulated by retinoic acid 8). Control cells treated with RA showed more induction (3.7-, 2.3-, and 4.6-fold change) compared to DKO+RA cells (2.8-, 1.2-, and 3-fold change) (Figure 5.8B). The reduced RA induction among primary response genes in *Hdac1/2* deleted ES (DKO) cells suggests a positive role of HDAC1/2 in regulating the expression of these genes.

A.



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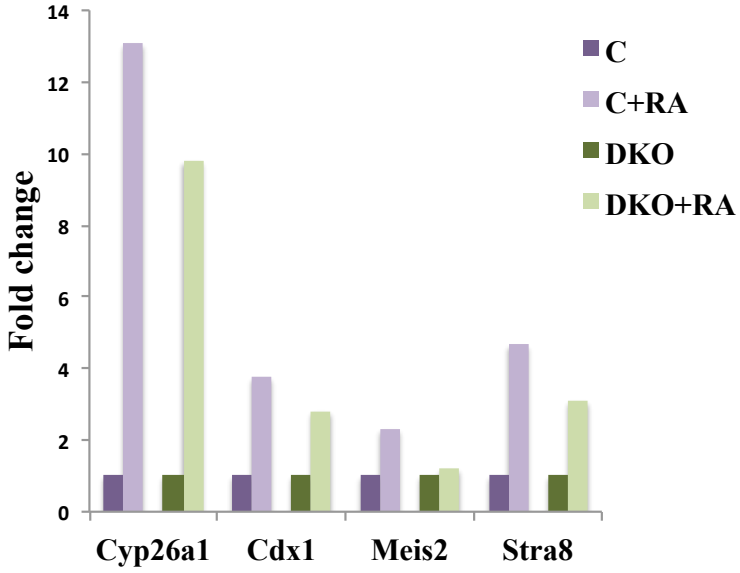

Figure 5.8: HDAC1/2 positively regulate expression of primary RA response genes. (A) Expression levels of HOX genes and (B) RA-primary response genes were compared between control treated with RA(C+RA) versus control and between DKO treated with RA (DKO+RA) versus KO. Fold change was calculated using microarray data.

As mentioned above, RA initiates gene expression by binding to RA receptors, which then binds RAREs within the promoters of target genes. To examine whether the reduction in the expression of RA target genes is a direct effect of Hdac1/2 deletion or RA receptor levels, I examined the expression levels of RARs and RXRs in control and DKO cells. The expression level of RAR and RXR receptors was unchanged between control and DKO cells (Figure 5.9 A and C). RARβ, which is primary RA responsive gene, was induced by RA treatment, although its induction was also reduced by the absence of HDAC1/2 (C+RA versus DKO+RA).

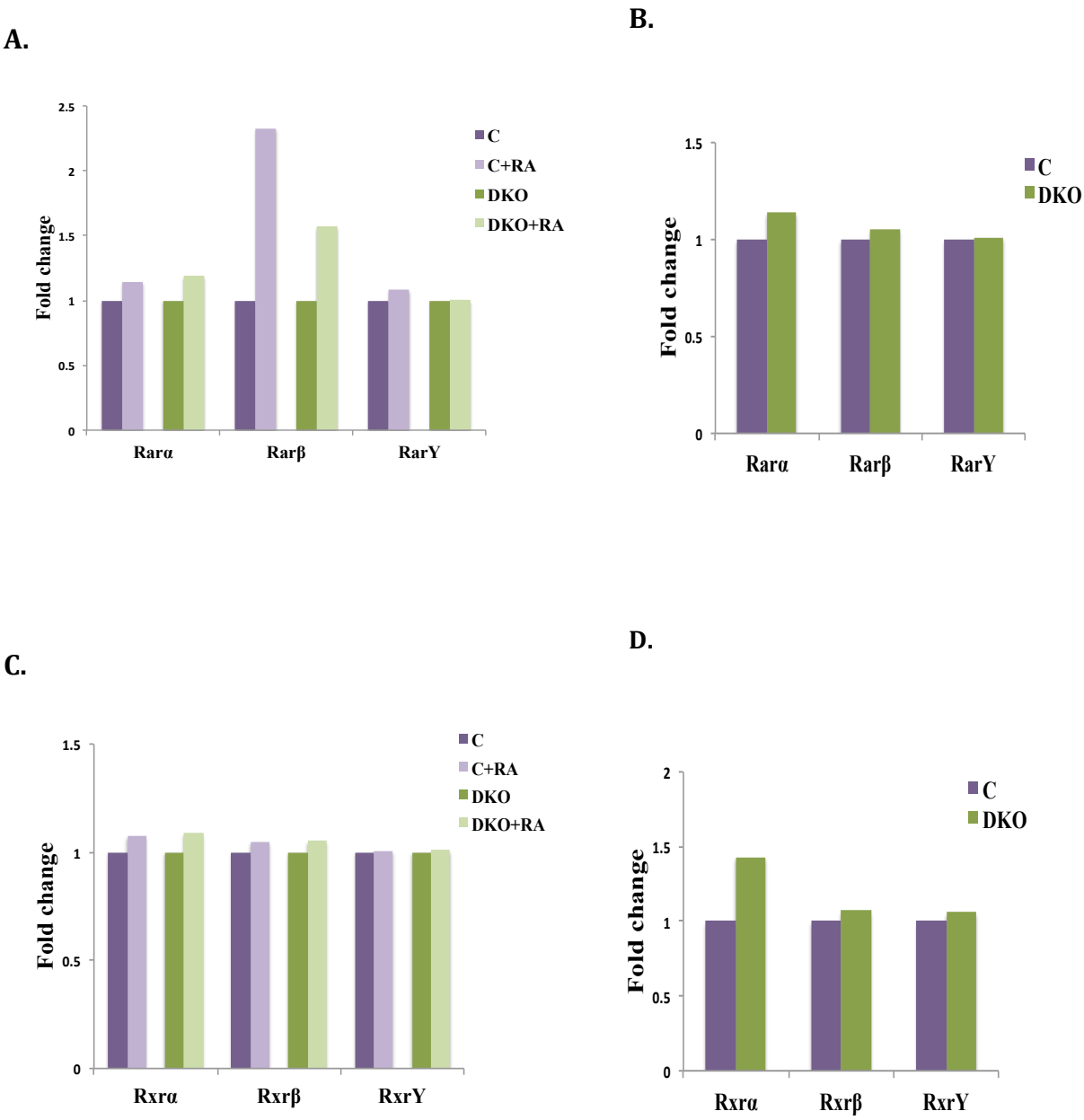

Figure 5.9: Expression of RA receptors is unchanged in *Hdac1/2* deleted cells. Expression levels of (A) retinoic acid receptors RARs and (B) retinoic x receptors RXRs compared between control treated with RA (C+RA) versus control and between DKO treated with RA (DKO+RA) versus DKO (C and D) between C (control) versus DKO (OHT treated). Fold change was calculated using microarray data.

Furthermore, the transcription levels of the top one hundred up-regulated and the top one hundred down-regulated genes (ranked based on fold changes) from the microarray data were compared between RA treated cells (C+RA versus DKO+RA), we also found that the pattern of gene expression is reduced in DKO+RA compared to C+RA (Figure 5.10). Among the one hundred up-regulated genes, *Aurkc* (a protein kinase) showed a reduced induction in DKO+RA compared to C+RA (4.5-compared to 1.5-fold). Moreover, the induction of *Tal2* and *Cdx1* were reduced by the absence of HDAC1/2 (3.1- , 3.6-fold in C+RA compared to 1.2-, 2.5-fold in DKO+RA). Out of the one hundred down-regulated genes, *Otx2* and *Fgf 8* showed less reduction in DKO+RA compared to C+RA (2.6-, 2.1-fold compared to 2-, 1.1-fold).

An analysis of functionally related genes groups among up-regulated genes comparing RA treated cells with untreated cells, revealed that genes involved in development, for instance, nervous system development, organ development and embryonic development were significantly up-regulated in control and DKO treated with RA (Figure 5.10).

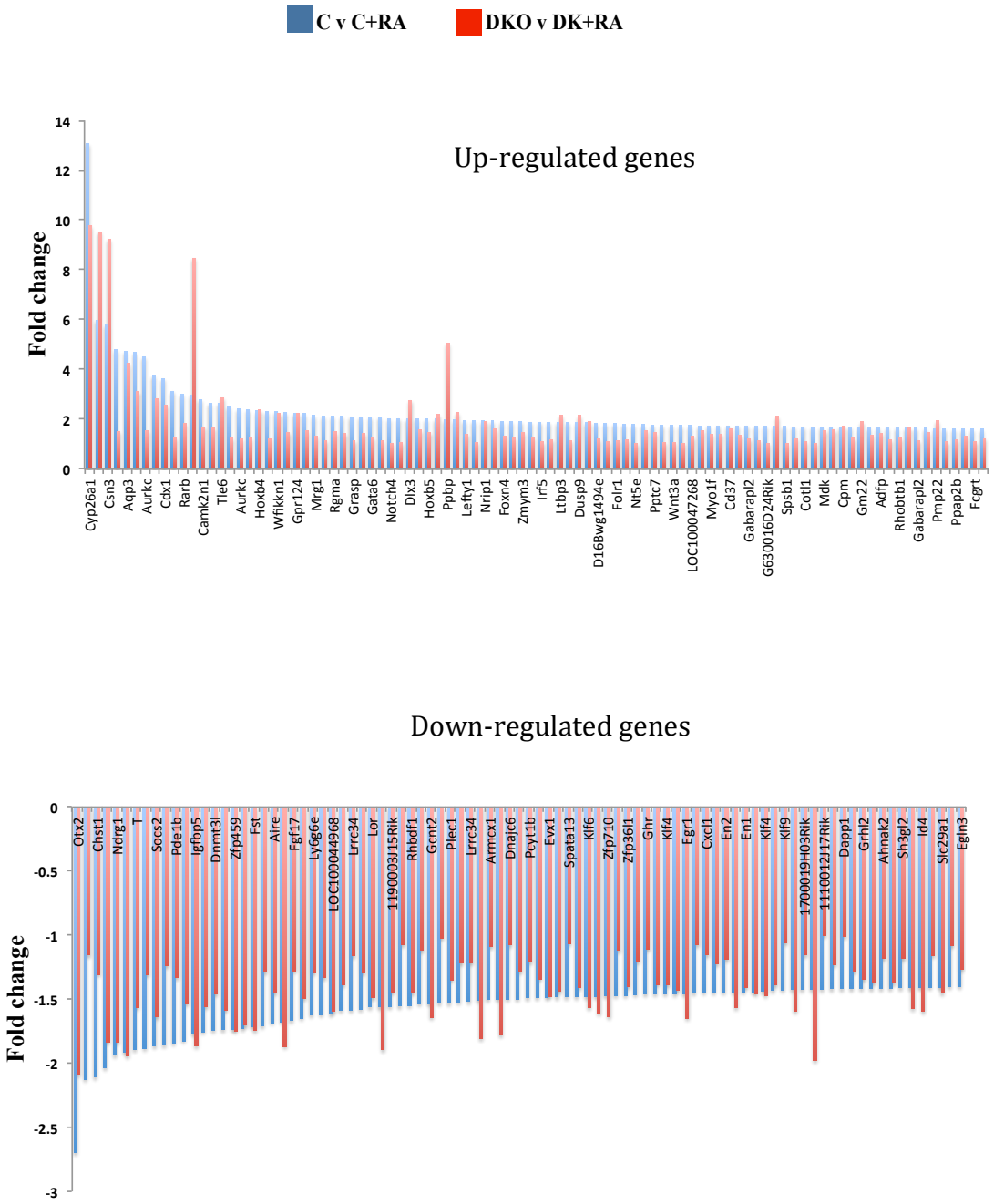

Figure 5.10 Comparative analysis of the top one hundred up- and down-regulated genes following RA treatment. Transcription levels were compared between control treated with RA (C+RA) versus control and between DKO treated with RA (DKO+RA) versus DKO. Genes ranked based on fold change.

5.3 Conclusions

In conclusion, *Hdac1*^{KO}*; Hdac2*^{Het} KO ES cells were able to form EBs over a two-day period. However, the EBs were irregular, reduced in size (Figure 5.1) and showed increased expression of cardiomyocyte-specific markers (mesodermal linages) (Figure 5.2 and 5.3). Whereas, differentiation of *Hdac1*^{KO}*; Hdac2*^{Het} KO ES cells in LIF- free and Serum-free medium toward neuroectoderm linage suggested the differentiation is unaffected, the expression of two important neuroectoderm linage transcription factors, SOX1 and PAX6, were unchanged in *Hdac1*^{KO}*; Hdac2*^{Het} cells compared with undeleted control (Figure 5.4).

HDAC1 and HDAC 2 appear to positively induce the expression of RA primary response genes, particularly HOX genes. Comparative microarray analysis of *Hdac1/2* deleted cells treated with RA (6hours) revealed that, although transcript levels of HOX genes (RA direct gene response) increased, it was to a lesser extent than in control cells (Figure 5.9A). Moreover, induction of RA primary response genes were reduced in *Hdac1/2* deleted cells in compared to control cells (Figure5.9B). These results suggest that HDAC1/2 are involved in gene activation as well as gene repression.

Chapter 6: Discussion

6.1 HDAC1 and HDAC2 are the dominant deacetylases in ES cells.

To assess the requirement of HDAC1 and HDAC2 in early embryogenesis, we have generated ES cells in which both copies of *Hdac1 (KO)* and a single *Hdac2 (Het)* gene can be inactivated conditionally (Figure 3.1). The total deacetylase activity of the cell decreased by approximately 56% three days after gene inactivation (Figure 3.3). Dovey et al. and Lagger et al. indicated a reduction in the deacetylase activity in their $Hdac1^{-/-}$ ES cells (Dovey, O.M. et al, 2010, Lagger, G., et al., 2002). However, in $Hdac1^{KO}$; $Hdac2^{Het}$ cells the level of HDAC2 protein is only slightly reduced and did not compensate for the loss of HDAC1, which is presumably due to having only a single copy of the *Hdac2* allele (Figure 3.2). Loss of both copies *Hdac1* and *Hdac2* (DKO) in ES cells (chapter 4) also results in a 60% reduction in the cellular deacetylase activity (Figure 4.2) despite a compensatory increase in the protein level of HDAC3, a highly related class I HDAC (Figure 4.7A). Concordant with these results, the level of direct HDAC1/2 binding partners (Sin3A, CoREST and MTA2) were significantly reduced (Figure 3.4 and 4.7). These results indicate that HDAC1 and HDAC2 are the dominant deacetylases in the ES cells, and their loss disrupts co-repressor complex integrity. Even though the deacetylase activity is significantly reduced, we observed a relatively modest increase in the acetylation levels of lysines within the tails of H3and H4 (Figure 3.5 and 4.8). A plausible explanation for this result is the fact that ES cells maintain a relatively plastic chromatin structure, to have the capacity to enter multiple distinct differentiation pathways as directed by the correct signals. Therefore, it shows a relatively high basal level of histone acetylation.

6.2 Loss of HDAC1 and HDAC2 causes defective chromosomal segregation and loss of cell viability.

HDAC1 and HDAC2 have been implicated in cell cycle regulation, they are required for transcription repression mediated by retinoblastoma tumor suppressor protein, Rb (Brehm, A., et al 1998), and they control expression of specific CDK inhibitors such as $p21^{Cdkn1a}$ (Lagger, G., et al., 2002 and Senese, S., et al., 2007, and Zupkovitz G., et al., 2010). The Proliferative ability of $Hdac1^{KO}$; $Hdac2^{Het}$ cells is not effected (Figure 3.6), however, deletion of both *Hdac1/2* (DKO) results in a profound loss of cell viability (figure 4.3) with 75% sub G1 cells, implying that a reduction in gene dosage negatively effects ES cell viability, since cells retaining only a single copy of the *Hdac2* allele are viable. Finally, it also suggests functional redundancy between HDAC1 and HDAC2.

If DKO cells are stimulated to exit cell cycle before deletion of both *Hdac1/2*, by making embryoid bodies or using retinoic acid (RA), the majority of cells remained viable, suggesting that the lethality is cell cycle dependent. We observed a significant increase in the number of cells with segregation defects and monopolar spindles. Moreover, we detected a significant increase in DNA abnormalities in DKO cells compared to *Hdac1-KO*, and *Hdac1-KO; Hdac2-Het* cells but not *Hdac2-KO* cells (Figure 4.6). Comparing the HDAC activity of these cell lines revealed a significant reduction in HDAC activity in all but *Hdac2-KO* cells, suggesting that DNA abnormalities are dependent on the dosage of HDAC activity.

HDAC1 has previously been implicated in reduced proliferation in ES cells (Lagger et al. 2002). Deletion of *Hdac1/2* in MEFs results in growth arrest and cell cycle block

in G1-phase that is associated with up-regulation of cell cycle inhibitors p21 and p57 (Yamaguchi, T., et al., 2010 and Wilting, R., et al., 2010). Loss of cell proliferation is a common phenotype in all *Hdac1/Hdac2* knockout, knockdown and HDAC inhibitors studies (Yamaguchi T., et al., 2010 Wilting R.H., et al., 2010, and Zupkovitz G., et al., 2010), a phenotype associated with up-regulation of cyclin-dependent kinase (CDK) inhibitors $p21^{Cdkn1a}$ that limits G1-to S-phase transition. Therefore, HDAC activity is crucial during this regulatory G1 phase. However, our analysis of DKO cell cycle revealed no obvious arrest before cell death occurred (Figure 4.3C), which is likely due to the fact that ES cells have a short G1 phase (1.5 hours), in which CDK2 complexes are constitutively active and RB hyper-phosphorylated (Budon, T., et al. 2002, Savatier, P., et al.1994). Therefore, DKO ES cells lacking the normal G1 regulatory step observed in somatic cells , are unable to arrest in G1 phase and consequently enter S phase and then mitosis where the absence of HDAC1/2 activity causes lethality. The significant increased in both chromatin bridges and micronuclei in DKO cells, suggests that deletion of HDAC1/2 leads to DNA replication defects. This is supported by a data from Sirbu et al. who used the iPOND (isolation of proteins on nascent DNA) and found that HDAC1/2 are present at active replication forks (Sirbu, B., et al., 2001). Moreover, a recent study has found that knockdown or chemical inhibition of HDAC1/2 reduced replication fork velocity and activates the replication stress response (Bhaskara, S., et al., 2013). Another study has also demonstrated that the deletion of other HDAC1/2 co-repressor components, including SDS3 in MEFs and Sin3A in *S. Pombe* impairs chromosome segregation and formation of pericentric heterochromatin, implying that HDAC1/2 activity maintain hypoacetylated state of pericentric heterochromatin, a requirement for appropriate assembly of the kinetochore

(David, G., et al., 2003; Silverstein, R., et al., 2003). We therefore conclude that a combination of DNA replication and mitotic defects are the major cause of death in DKO cells.

6.3 HDAC1/2 regulate expression of core pluripotency factors in ES cells

A reduction in the expression level of pluripotent factors was detected in both *Hdac1*^{KO}*; Hdac2*^{Het} and DKO cells, implying that loss of HDAC1/2 activity correlates with the reduced expression of pluripotent factors (Figure 3.10, 3.11 and 4.11). Interestingly, this phenotype contrasts with the disruption of other HDAC1/2-containing complexes in ES cells. Deletion of LSD1 (lysine demethylase 1) perturbs the CoREST complex but does not effect expression of Oct4 (Foster, C., et al., 2010). Conversely, deletion of MBD3, a component of the NuRD complex, prevents repression of Oct4 (Kaji, K., et al., 2006). However, the Sin3A-HDAC complex was found to positively regulate expression of Nanog in ES cells (Baltus, G., et al., 2009). Moreover, A recent genome-wide analysis (ChIP-chip) revealed binding of HDAC1 to active genes in ES cells, including core pluripotent factors Oct4, Nanog and SOX2 (Kidder, B., et al., 2011), suggesting the positive role of HDAC1 in maintaining self-renewal of ES cells.

ChIP analysis has been used to map the genome-wide binding sites of HDAC1 and reveal enrichment of HDAC1 binding to active gene loci (Kurdistani, S., et al., 2002; Wang, Z., et al., 2009). Altogether, HDAC1/2 are necessary for the expression of pluripotent factors, which also revealed the positive role of HDACs in transcription and change the view of HDAC1/2 as repressive factors.

6.4 Inositol tetraphosphate (IP4) regulates activity of HDAC1 in vivo

The cellular requirement for HDAC1 and HDAC2 in cell division and to influence gene expression is dependent upon deacetylase activity. For instance, we found a correlation between the reduction in deacetylase activity over time and the number of deregulated genes in DKO ES cells (Figure 4.9B). The increase in DNA abnormalities is also dependent on the dosage of HDAC1/2 deacetylase activity (Figure 4.7). We were able to demonstrate an essential requirement of the HDAC1/2 activity using rescue experiments (section 4.2.8). The lethal phenotype of DKO ES cells could be rescued by transfection with cDNA for a wild-type HDAC1, while a catalytically inactive HDAC1^{Y303H} was unable to rescue the cell viability and the cells died at day 4 following *Hdac1/2* deletion (Figure 4.13B).

Recently, it has been shown that the deacetylase activity of HDAC1 and HDAC3 is regulated through binding of IP4 molecules, sandwiched between the HDAC and its cognate corepressor, in a highly basic pocket (Millard, C., et al 2013, Watson, P., et al. 2012). This finding raised an important question as to whether IP4 regulates HDAC activity in vivo as well. Therefore, DKO cells were used as model system to test the requirement for IP4 binding to the activity of HDAC1. Substitution of the positively charged residues in the IP4 binding pocket , essential for IP4 binding to a polar non-charged Glutamine (K31Q, R270Q, and R306Q) reduced the deacetylase activity of HDAC1 and also its ability to rescue the viability of DKO cells. The double mutants (K31Q/R270Q and R270Q/R306Q) showed a lower activity compared to the individual whereas the triple mutants (K31Q/R270Q/ R306Q) had lowest deacetylase

activity of all and the smallest number of viable cells (Figure 4.14 and 4.15), implying that mutations that prevent IP4 binding reduced the activity of HDAC1 in vivo.

6.5 Deletion of HDAC1-KO; HDAC2-Het predisposes cardiac differentiation of ES cells.

It has been previously shown that, ES cells lacking MBD3 exhibit a differentiation defect due to inability to repress Oct4 gene (Kaji, K., et al., 2006). However, disruption of the NODE complex, which contains similar core components to NuRD complex but lacks MBD3, leads to increased differentiation. Knockdown of MTA1 causes activation of expression of endodermal-specific markers (GATA6 and Foxa2) (Liang, J., et al., 2008). Inactivation of $Hdac1^{KO}; Hdac2^{Het}$ does not inhibit the differentiation capacity of ES cells since AP staining is lost upon removal of LIF (Figure 3.7). Moreover, $Hdac1^{KO}; Hdac2^{Het}$ ES cells were able to switch off expression of core pluripotency factors (Oct4 and Nanog) when induced to differentiate using different methods, including withdrawal of LIF, generating EBs and using N2B27 serum-free media (Figure 3.11, 5.2 and 5.4), suggesting that the potential of ES cells to exit the pluripotent state is not inhibited by deletion of $Hdac1^{KO}; Hdac2^{Het.}$.

Dovey et al, demonstrated that deletion of HDAC1 causes precocious differentiation of ES cells identified by elevated expression of cardiomyocyte and neural markers in EBs (embryoid bodies), whereas, HDAC2-deficient cells were similar to controls (Dovey, O., et al., 2010). $Hdac1^{KO}; Hdac2^{Het}$ cells were able to form EBs, however, from day 4 EBs derived from $Hdac1^{KO}; Hdac2^{Het}$ were irregular and reduced in size compared to undeleted controls, suggesting the presence of increased differentiation (Figure 5.1 A

and B). This might be expected because EBs lacking HDAC1 showed a similar phenotype (Dovey, O., et al., 2010), and both cell types had a significant reduction in the deacetylase activity. Moreover, analysis of the transcriptional profile by particularly examined cardiomyocyte-specific marker, revealed that EBs lacking *Hdac1*^{KO}*; Hdac2*^{Het} were predisposed to differentiate toward cardiomyocyte (mesodermal) lineages. The expression levels of early cardiomyocyte markers (*Nkx2-5*, *Mef2c)* and late cardiomyocyte markers (*TBX 5*, *TNNT2*) were increased in *Hdac1*^{KO}*; Hdac2*^{Het} EBs (Figure 5.2), suggesting that reduction in deacetylase activity promotes cardiomyocyte differentiation under the growth condition used. This result is consistent with a number of studies, in which treatment of day 7 EBs with TSA (an HDAC inhibitor) for 24 hours induced expression of Nkx2.5, which indicates cardiomyocyte differentiation (Kawamura, T., et al., 2005). A further study showed that the WNT signaling pathway promotes expression of Nkx2.5 by downregulation of HDAC1, which consequently induced cardiomyogenesis (Liu, Y., et al., 2009). In contrast, induced differentiation of ES cell toward neuroectodermal lineage is unaffected in HDAC1^{KO}; HDAC2^{Het} cells. We used LIF-free/Serm-free (N2B27) media to induce neuroectodermal differentiation in HDAC1^{KO}; HDAC2^{Het} ES cells. Expression of neuroectodermal markers (SOX1 and PAX6) were induced, implying that HDAC1^{KO}; HDAC2^{Het} ES cells have successfully entered the neuronal lineage and exited the pluripotent state, as Nanog was repressed by day 2 (Figure 5.6). Moreover, we observed no differences in the level of additional neuronal markers, including ASCL1, CXCR4, FZD9, and Noggin (Figure 5.6).

Collectively, gene expression data indicate that, the cardiomyocyte markers Nkx2-5 and Mef2c are regulated by HDAC1/2 since loss of $HDAC1^{KO}$; $HDAC2^{Het}$ increased their expression and consequently promotes cardiomyogenesis, whereas, HDAC1/2 are non-essential for neuronal lineage since the neuroectodermal markers are not significantly effected in $HDAC1^{KO}$; $HDAC2^{Het}$ ES cells under these growth conditions.

6.6 HDAC1/2 positively regulate expression of HOX genes

Inactivation of HDAC1/2 in ES cells (DKO) results in deregulation of almost 2,000 genes, with a correlation between the reduction in deacetylase activity and the number of deregulated genes (Figure 4.9A). By day 3, 994 transcripts were down-regulated, suggesting that HDAC1/2 may have a role in maintaining the expression of some genes, in addition to their well characterized role in gene repression (Figure 4.9B). As already discussed, among these down-regulated transcripts were the pluripotency factors Oct4 and Nanog (Figure 4.9 C and 4.11). Genome-wide ChIP studies that mapping binding sites of HDAC1 in human and Rpd3 in yeast revealed enrichment of HDAC1/Rpd3 at active gene loci and are positively associated with gene transcription, in which they positively correlate with pol II levels, mRNA expression levels and interestingly with histone acetylation levels (Wang, Z., et al., 2009 ; Kurdistani, S., et al., 2002).

Treatment of ES cells with retinoic acid is a simple and effective way of activating a well characterized gene expression programme. Within 6 hours approximately 200 genes are induced and therefore we hypothesized that any changes in the expression of these genes in the absence of HDAC1/2, should be a direct transcriptional effect.

Therefore, to identify direct effects of HDAC1/2 on gene expression, HDAC1/2 deleted cells (DKO) were treated with RA (for 6 hours) that rapidly induces expression of target genes. Treatment of DKO ES cells (day 2.5 post OHT treatment) with RA results in a change in the expression of 195 genes (Figure 5.7, compare DKO versus DKO+RA) compared to 238 genes in the control (Figure 5.7, compare C versus CRA), suggesting that loss of HDAC1/2 is affecting the expression of these genes. Addition

of RA to ES cells (control) induces transcription of RA primary response genes, in particular HOX genes (2-3 fold change at 6 hours) and *CYP26* (13-fold), which mediates RA catabolism (Figure 5.9). However, addition of RA to DKO cells did not induce the expression of these genes to same extent as detected in control cells (Figure 5.9). *CYP26a* was induced by only 7.9-fold at 6 hours in DKO+RA. The expression levels of *Hoxa1*, *Hoxa5* were significantly induced in C+RA (2.9- and 2.3-fold), compared to only 1.2-fold change in DKO+RA. Furthermore, the induction of *Hoxb1* and *Hoxb5* were reduced in DKO+RA (1.1- and 1.4-fold) compared to (2.2- and 2-fold) in C+RA. The gene expression pattern was also reduced in DKO+RA compared to C+RA (Figure 5.11). All these data implying that, loss of HDAC1/2 effect induction of RA target genes, particularly HOX genes. A recent study has reported the binding of HDAC1/2 to various RAREs in the promoter or enhancer of RA target genes (Urvalek, A., and Gudas, L., 2014), which suggests that HDAC1/2 are directly regulating their expression. HDAC1 has been shown to be co-activator for the glucocorticoid receptor (GR) and this function is dynamically regulated by acetylation of C-terminal tail of HDAC1 which regulate its deacetylase activity (Qiu, Y., et al., 2006). Collectively these data indicate positive role of HDAC1/2 in gene activation as well gene repression.

6.7 Summary

In this thesis, we have shown that HDAC1/2 are required for the integrity and full deacetylase activity of the HDAC1/2- corepressor complexes. Loss of HDAC1/2 leads to deregulation of almost 2,000 genes including a down-regulation of the core pluripotent factors, Oct4 and Nanog, suggesting essential role of HDAC1/2 in regulation of stem cell self-renewal. The deacetylase activity of HDAC1/2 appeared to be required for the induction of HOX genes, which demonstrate the positive role of HDAC1/2 in regulating gene expression. We also demonstrated that the binding of IP4 is necessary for the full activity of HDAC1 vivo.

Inactivation of *HDAC1/2* resulted in loss of ES cell viability due to defects in DNA replication and mitosis, which suggest that blocking deacetylase activity using specific inhibitors of HDAC1/2 could potentially be an effective therapeutic strategy for the treatment of cancer.

Appendices

Table 1: List of antibodies

Antibody	Clonality	Source	Dilution	Company	Product Code
Hdac1	Polyclonal	Rabbit	1:2000	Santa Cruz	SC-7972
Hdac2	Monoclonal	Mouse	1:2000	Millipore	05-814
Hdac3	Monoclonal	Rabbit	1:2000	Abcam	Ab32369
Hdac8	Monoclonal	Mouse	1:2000	Abcam	Ab12176
mSin3a	Monoclonal	Rabbit	1:2000	Abcam	Ab129087
MTA-2	Monoclonal	Mouse	1:2000	Sigma	M-276
SDS3	Polyclonal	Goat	1:2000	Bethyle	A300-235A
LSD-1	Polyclonal	Rabbit	1:2000	Abcam	Ab37165
Oct4	Polyclonal	Rabbit	1:500	Abcam	Ab19857
Nanog	Polyclonal	Rabbit	1:2500	Bethyle	A300-397A
α-Tubulin	Monoclonal	Mouse	1:5000	Sigma	TC168
H3	Monoclonal	Mouse	1:2000	Milipore	05-499
H3K9ac	Monoclonal	Rabbit	1:2000	Milipore	04-1003
H3K14ac	Monoclonal	Rabbit	1:2000	Millipore	04-1044
H3K18ac	Monoclonal	Rabbit	1:2000	Milipore	04-1107
H3K23ac	Polyclonal	Rabbit	1:2000	Active Mtif	39132
H3K27ac	Polyclonal	Rabbit	1:2000	Active Motif	39135
H3K36ac	Polyclonal	Rabbit	1:2000	Milipoore	07-540
H3K56ac	Polyclonal	Rabbit	1:2000	Active Motif	39281
H4K5ac	Polyclonal	Rabbit	1:2000	Active Motif	39699
H4K12ac	Polyclonal	Rabbit	1:2000	Active Motif	29927
H4K16ac	Polyclonal	Rabbit	1:2000	Active Motif	39167
PARP	Polyclonal	Rabbit	1:1000	Cell Signaling	4592
Caspase 3	Polyclonal	Mouse	1:5000	Cell Signaling	9668

Table 2: List of primers and Universal Probe Library (UPL) hydrolysis probe used for qRT-PCR

Gene	Universal Probe Library primer sequence	UPL hydrolysis probe	Size (bp)
Nanog	L gcctccagcagatgcaag R ggttttgaaaccaggtcttaacc	91	75
Rex1	L ttctctcaatagagtgagtgtgcag R aggcgatcctgctttcttct	33	68
Pou5f1	L aatgccgtgaagttggagaa R ccttctgcagggctttcat	95	70
Ccdn2	L caccgacaactctgtgaagc R tccacttcagcttacccaaca	17	71
Gdf3	L gggtgttcgtgggaacct R ccatcttggaaaggtttctgtg	7	78
Eif5	L cgcgttgggtttatgtcttt R gctatgtttccccaatacaggt	46	77
Amnionless	L tacgagacagtcacgccatc R gaggccaggaccaactcc	34	64
Camk2n2	L ccagtctgcccaattctga R gataccttgggagggaggagt	79	61
Adssi1	L aaggccgtgtcattcattg R tcagccctttcttctcgttc	13	88
Thy1	L aactcttggcaccatgaacc R tcaggctggtcaccttctg	15	89
Ly6a	L aaggtcaacgtgaagacttcct R cctccattgggaactgctac	72	56
Blvrb	L cgatgtggacaagactgtgg R tcggacattactgtagtgggact	104	90
HMGN3	L gcaaatggtgacactaaagttga R ttccacgacaattcactctcc	98	78
Med7	L tgggataagaaatcggcaaa R tgaagatgacaaggaaccaaaa	7	72
Pfn1	L ctgtcaccatgactgccaag R gatcaaaccaccgtggaca	18	68
Tcf25	L ctcaccatgttccctggagt R catcaggtcgcacactgc	67	61
MTA2	L ccgaagaccctatgcaccta R agccttaggaagtcggatcg	13	70
Nestin	L tgcaggccactgaaaagtt R ttccaggatctgagcgatct	2	89
GPS1	L gcaggaagatccgcagaa R ccactgtagctggctgcata	22	90

ASCL1	L gacctgccaggctctcct R cgttggcgagaaacactaaag	38	71
CXCR4	L tggaaccgatcagtgtgagt R gggcaggaagatcctattga	38	70
FZD9	L tttcttctccacggccttc R ggtactggaaccggtgagg	4	62
Noggin	L tgatggatccccaccaac R cgctagagggtggtgaaact	10	66
Pax6	L gttccctgtcctgtggactc R accgcccttggttaaagtct	78	61
SOX1	L gtgacatctgcccccatc R gaggccagtctggtgtcag	60	60
Cyp26a1	L ccggcttcaggctacaga R ggagctctgttgacgattgtt	17	125
CDX1	L acgccctacgaatggatg R cttggttcgggtcttaccg	70	72
Meis2	L agacaaggacgcaatctatgg R gctcgcacttctcaaaaacc	6	68
Hoxa1	L agaaaccctcccaaaacagg R ttgttgaagtggaactccttctc	78	122
Hoxa2	L agaaggcggccaagaaaa R catcagctatttccagggattc	70	93
Hoxa5	L agctgcacattagtcacgaca R gcggttgaagtggaattctt	1	110
Hoxb1	L aagagaaacccacctaagacagc R tgaagtttgtgcggagacc	33	76
Hoxb4	L ctggatgcgcaaagttcac R gtgaaactccttctccaactcc	62	118
Hoxb7	L ctggatgcgaagctcagg R ccgagtcaggtagcgattgta	1	109
Otx2	L gactgcagggcagagacg R ggtagatttggagtgacggaac	25	111
SP5	L aggacaggaaactgggtcgt R gatggctcggactttgga	79	60
Msrb2	L ggatcctgagacgactggac R aaacacatggccaaggtgag	10	89
Evx1	L tctcacgacccgacctgt R cttccctgccaatgtcaaac	7	62
Klf6	L tcccacttgaaagcacatca R acttcttgcaaaacgccact	2	90
Dapp1	L ctccaatggacgtgatggta R gaaagtgtttgacagagtctttgg	62	101
Nid2	L tgaccagcacacttgtatcttga R aggtgtgactgccatcgag	10	65
FGF5	L gagccctgaaggaaactcg R gcgaaacaaaatgacctgact	89	76
Tbx5	L cgaagtgggcacagagatg R caccttcactttgtaactaggaaaca	9	70

Tnnt2	L atgtctgacgccgaggag R ctgcctcctcttgctcgt	25	94
Myod	L ccaggacacgactgctttct R cacaccggctgtcctctac	52	76
Nkx2.5	L gacgtagcctggtgtctcg R gtgtggaatccgtcgaaagt	53	70
MEF2c	L tctgccctcagtcagttgg R cgtggtgtgttgtgggtatc	77	63
Gata4	L ggaagacaccccaatctcg R catggccccacaattgac	13	75

Table 3: List of genes used to assess pluripotent and differentiation state

Pluripotent Genes	Differentiation genes		
Bmp4	Afp	Hoxb2	Neurod2
Ccna2	Alb	Hoxb3	Nkx2-4
Cdc42	Amn	Hoxb4	Nkx6-1
Cfc1	Ascl2	Hoxb5	Nodal
Chd1	Cdh5	Hoxb6	Nog
Dppa1	Cdx2	Hoxb7	Otx2
Dppa2	Chrd	Hoxb8	Pax2
Dppa3	Cldn4	Hoxb9	Pax6
Dppa4	Dkk1	Hoxc10	Plac1
Dppa5	Dnmt3l	Hoxc11	Plac1l
E2f1	Eomes	Hoxc12	Plac8
Eed	Esx1	Hoxc13	Plac8l1
Ep300	Fabp1	Hoxc4	Smad2
Esrrbl1	Fgf5	Hoxc5	Smad3
Fgf4	Fgf8	Hoxc6	Snai1
Klf4	Foxa2	Hoxc8	Snai2
Klf5	Gata4	Hoxc9	Snai3
Lin28	Gata6	Hoxd1	Sox1
Mycbp	Gdf1	Hoxd10	Sox17
Nacc1	Gdf3	Hoxd11	Sox18
Nanog	Gfab	Hoxd12	Sox3
Nanogpd	Hand1	Hoxd13	Sox7
Nr0b1	Hand2	Hoxd3	Syp
Nr5a2	Hnf4a	Hoxd4	Sypl
Pou5f1	Hoxa1	Hoxd8	T
Prdm14	Hoxa10	Hoxd9	Tbx5
Sall4	Hoxa11	Irx3	Tbx6
Slc2a3	Hoxa11s	Lmna	Tead2
Smad1	Hoxa13	Mesp1	Tead3

Sox2	Hoxa2	Mesp2	Tead4
Stat3	Hoxa3	Mixl1	Tubb3
Suz12	Hoxa4	Msi1	Vim
Tcfcp2l1	Hoxa5	Msi1h	Wnt1
Tert	Hoxa6	Myh7	Wnt3a
Utf1	Hoxa7	Myod1	
Zfp296	Hoxa9	Ncam1	
Zfp42	Hoxb1	Ncam	
Zfx	Hoxb13	Nes	

Table 4: List of genes deregulated ≥1.4-fold (adjusted P < 0.05) in C vs. K(+LIF), C vs. K(-LIF), and C+LIF vs. C-LIF

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
Cbr3	2.252558277	Slc38a5	2.917329641
Gli2	2.174903663	Rbp1	2.785492067
Zfhx2	2.118774367	Amn	2.605649708
LOC208080	2.071353918	S100a6	2.59928191
Slc11a1	2.066920379	Mylpf	2.483426651
Phlda2	1.901891393	Ddx19b	2.404531434
Epb4.9	1.848924392	LOC100047651	2.366311058
AF067061	1.842900622	Gpx2	2.334610751
Notch4	1.814667991	Fabp3	2.332307462
Pitx2	1.758915444	Htra1	2.32703134
Hirip3	1.741823566	Taf9b	2.264709226
Gm1967	1.740600422	Cotl1	2.210645475
EG627299	1.732587424	1190020J12Rik	2.207514936
Nr5a2	1.7235139	Hmgn3	2.192903308
Ifitm3	1.722772529	1110008P14Rik	2.183438515
Ccnd1	1.721485238	Zbtb32	2.167550692
LOC675933	1.719290821	LOC381283	2.152935896
Phc1	1.717242535	Fgfr2	2.148313809
Pml	1.71692661	Slc30a3	2.136551007
Spp1	1.716329605	Taf7l	2.127520761
Frrs1	1.685341441	Ly6a	2.123575967
Rdm1	1.6810236	Cd74	2.107704924
Nanog	1.670167479	Rsph1	2.100641543
LOC381844	1.648351285	Slc5a5	2.082077266
2810474O19Rik	1.646048356	Adssl1	2.075417568
Nanogpd	1.641407458	Igf2	2.049937798
Ncor1	1.63844508	Myl4	2.028097501
2410081M15Rik	1.615890841	C3	2.010013761
5730528L13Rik	1.615643687	Plcd1	1.995415924
Dppa3	1.605073722	1700007E06Rik	1.994935036
Pus3	1.600503041	Ela2a	1.990884843
Senp3	1.599907012	Slc6a13	1.982733296
0610006I08Rik	1.595998356	Blvrb	1.97853189
Exosc5	1.587691635	LOC100046120	1.977286155
Jtb	1.580880072	Thy1	1.975608806

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
Ephx2	1.575690618	Guca1a	1.974439426
Rpp25	1.573158254	Rab25	1.944700451
LOC386199	1.572609487	Sct	1.923545322
Bckdha	1.549443468	Pmp22	1.923428749
Nupr1	1.546136322	Calml4	1.917381963
Zfp35	1.545982482	LOC677144	1.91264274
Tmem51	1.545599288	H19	1.910190049
Mettl4	1.545369297	Crxos1	1.898496298
Ssr2	1.544309772	2810003C17Rik	1.873706894
Bhlhb2	1.544190774	Cpm	1.867642017
LOC100043402	1.543075484	Chst1	1.858759385
Rnf113a2	1.542044044	Wnt7b	1.856287059
Isy1	1.534265492	Gpx3	1.856150116
Mid1ip1	1.530562566	Krtdap	1.854669969
4933434E20Rik	1.5290166	Crlf1	1.840744027
Zcwpw1	1.526486868	Ttc9b	1.837329407
Wdr43	1.526086947	Ptprs	1.835158277
Slc28a1	1.517627628	Atp12a	1.834794167
Nodal	1.5174405	Dusp4	1.815304709
Upp1	1.516822015	Actn2	1.811842408
Cobl	1.515830221	Tspan17	1.811491737
Sfrs5	1.515660264	Acss1	1.797965891
LOC383491	1.510996934	Tex19.2	1.797586625
Pank1	1.510481446	Pdlim4	1.794805886
Mia1	1.496234179	Wfdc2	1.791504636
Rrp1b	1.494010107	Dkk3	1.781640009
Actn3	1.492172231	Cib2	1.774529498
Ecd	1.489871095	Pyy	1.773028446
Hspbap1	1.489760618	Gstk1	1.772117809
BC085271	1.488851454	4933421H10Rik	1.770682119
Zscan4c	1.485657149	Camk2n2	1.767516129
Yap1	1.484107604	Fbln2	1.763588161
Hsd17b1	1.482595005	Spink2	1.75970862
Rabif	1.482572619	Fbxo2	1.754887174
Msi2	1.481755339	Anxa5	1.743251855
Bxdc2	1.481235725	H2-Bl	1.742384379
Cwf19l2	1.480864026	A530057A03Rik	1.740952791
Dnajc7	1.480711831	Bmp1	1.739382029
Myst4	1.479124123	Crabp2	1.737393132
Mtrf1	1.477516986	Sstr2	1.735929003
Sap30	1.477457488	Coro1a	1.728199348
Ifitm1	1.476291784	Tes	1.719508317
2410137M14Rik	1.475252865	EG630499	1.71726083
Def6	1.471121327	Anxa2	1.716806627
LOC381140	1.470493292	Pdzk1	1.710240005
Med6	1.469151374	Myl2	1.707361581
BC088983	1.468227871	Tppp3	1.706465207
Dtymk	1.467876439	2310022B05Rik	1.700949723
Dus4l	1.467231486	Eml1	1.692755639
D4Wsu132e	1.46567256	Lgals3	1.690956058
M6pr	1.465177322	Ypel5	1.690525675
2310001H12Rik	1.461726689	Gas6	1.686708952
2610207P08Rik	1.460851678	Lbh	1.685410458
Mrps18b	1.460485721	Csrp1	1.685260693

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
2600005C20Rik	1.453167932	Ostm1	1.678942509
Kdelc1	1.452729369	Stxbp1	1.668596431
Bcap29	1.451683644	LOC100044190	1.66601333
Deadc1	1.451395147	Ctnnal1	1.664970687
Mtbp	1.447458097	Mapk13	1.664816716
LOC638892	1.444830028	Fam115c	1.661878804
Tmem39a	1.444171577	Espn	1.660262616
Dhdh	1.443811339	Gng13	1.658856663
Zfp292	1.443774565	LOC100045542	1.65867347
Ppp1r8	1.443606456	Acadvl	1.657224075
Tmem92	1.443034702	Npw	1.654908225
Rnaseh2b	1.442353952	Stard10	1.654265208
1110002D22Rik	1.441622455	Lypd2	1.650720081
Gm428	1.437675068	Ndrl	1.649990089
Tut1	1.435851004	Stag3	1.649963147
Silg111	1.435207513	Sdc3	1.649586963
Mrpl17	1.434747678	Ckb	1.649519721
Kti12	1.432076413	LOC100045981	1.64839309
Hrmt1l2	1.432058524	Cyba	1.642092804
Nudt5	1.42932167	Prkcb	1.640091678
Hvcn1	1.428697211	Pip4k2c	1.636391783
Sfrs2ip	1.42582193	1700027N10Rik	1.633240529
Hdgf	1.423409765	Casp9	1.631365406
Tcstv3	1.422446648	Rtn1	1.62700379
Jarid1b	1.419728893	Smarca2	1.62628679
Ech1	1.419262985	9130213B05Rik	1.62299977
E330016A19Rik	1.41826458	Cuta	1.621046852
Prmt6	1.417424241	Lamb3	1.616051454
Zscan10	1.417194839	Micall2	1.613412874
3110009E18Rik	1.414952956	Dbndd2	1.611436518
Cugbp1	1.414164189	Mgst3	1.607444834
1110039B18Rik	1.413999794	Tdrkh	1.605947656
Alkbh3	1.411205552	Rgs10	1.604990355
Epdr1	1.410968228	Phc2	1.60437958
Sulf1	1.410800248	Cnn2	1.603810025
Gdf15	1.408640134	Gstt1	1.60374352
Ogt	1.408511414	Gstt3	1.603127254
Zfp219	1.407641665	Nid2	1.600860883
Ubl4	1.406691334	Reep5	1.599625279
Hes1	1.406615719	1700016K19Rik	1.598257542
Zfp239	1.404993426	LOC100048733	1.595352365
BC019806	1.40285662	Tmem45b	1.593128411
Utx	1.401889818	Bgn	1.592131715
Use1	1.401731881	Arpc1b	1.59098891
		LOC100047937	1.589890527
		Mt1	1.586633403
		Nrip3	1.584094843
		Dctn3	1.582850895
		Prkcz	1.582115422
		Spag6	1.581722878
		LOC674135	1.581203667
		Mfge8	1.578893359
		Cldn10	1.576223228
		Slc24a6	1.576157599

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
		Gpsm1	1.574681472
		Fam131a	1.573581541
		Klrg2	1.573150038
		Sema7a	1.569227215
		Mfsd7c	1.566968361
		Gm1673	1.566835468
		Copz2	1.564597734
		Eno2	1.564179973
		Ptrf	1.562972341
		Nudt4	1.562799547
		Tmem130	1.56154645
		2700060E02Rik	1.561109621
		Slc17a7	1.556926453
		Cstb	1.555866518
		Ctsh	1.555260084
		Psmb9	1.554432986
		Col4a2	1.55392876
		Akr7a5	1.55372263
		Acss2	1.552265051
		Col4a1	1.551040362
		LOC671878	1.550441951
		Stat3	1.549654407
		Insl6	1.548611666
		D330028D13Rik	1.54800793
		Tspo	1.546138748
		Nr6a1	1.543022836
		Pja2	1.542239923
		Mylk2	1.540150757
		Mtch1	1.53911295
		Cd63	1.538553772
		1700088E04Rik	1.538410234
		Tax1bp3	1.537619121
		Pcgf5	1.535797823
		Rpl3l	1.533972218
		Rell1	1.53384979
		Psors1c2	1.533664126
		Nuak1	1.530880824
		Cyp4f14	1.530691577
		Aldh3a1	1.529885652
		Llgl2	1.529784384
		Mov10l1	1.528385135
		Cldn6	1.527841369
		Tceal5	1.527777818
		Rap2ip	1.52607148
		Phf13	1.52571953
		Usp2	1.524474659
		Edem2	1.52176728
		5031439G07Rik	1.521140595
		Gltp	1.520589546
		1700052O22Rik	1.52002232
		Wfdc10	1.519202125
		Gchfr	1.518063233
		Rhox6	1.514741714
		Rab3d	1.514739227

C vs. K (+LIF)			
Gene Symbol	FC down-regulated	Gene Symbol	FC up-regulated
		Plat	1.513479205
		Piwil1	1.513317661
		Fa2h	1.511965506
		Defb36	1.510138335
		LOC270344	1.508482857
		Maged2	1.507819603
		Jak2	1.504878044
		Matn1	1.503808749
		Trp53inp2	1.502587253
		Als2	1.502366167
		Podxl	1.500295383
		Lrp10	1.500149823
		Nphp4	1.50014204
		Stac2	1.498146945
		Dner	1.49739449
		Ostf1	1.496412362
		BC021614	1.49639995
		Ctsb	1.493927415
		Crym	1.493870184
		Nagk	1.493484495
		Tex101	1.493306344
		Prmt2	1.493170042
		1700001C19Rik	1.492843876
		Slc29a4	1.492124692
		Idh1	1.49208586
		B2m	1.492028178
		Rbp7	1.491779444
		Bik	1.491345852
		Calu	1.486391284
		Synpo	1.486325284
		App	1.485489039
		Krt7	1.485479672
		Sema6b	1.484325923
		Pkd2l1	1.484019336
		3110001A13Rik	1.483398107
		Psap	1.482851188
		Ngfr	1.481949253
		Anxa6	1.48123911
		Tmem9b	1.479455832
		Map1lc3a	1.479206166
		Gprc5a	1.478944912
		Atp6v0d1	1.477976977
		Epb4.1l2	1.476790434
		Slc9a3r2	1.476266703
		Tcf19	1.476178912
		Asphd2	1.474901693
		Tmem53	1.471442328
		AA407659	1.469376538
		Dync2li1	1.468445231
		Col18a1	1.468200447
		Col5a1	1.46730735
		Slc39a11	1.464079031
		0610007P22Rik	1.462970326
		8430427H17Rik	1.460998689

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
		Col7a1	1.460899169
		LOC100045780	1.460553186
		H2-DMa	1.460371577
		Vill	1.45992432
		Dnajc12	1.459157514
		Spag1	1.458762873
		Sccpdh	1.458292277
		Alox5ap	1.457722779
		Tesc	1.45630731
		D16H22S680E	1.45531432
		Plac8	1.454309036
		Cd79b	1.453945119
		2510002J07Rik	1.453039589
		Limk1	1.4512258
		Cmtm8	1.449904982
		Aacs	1.449544596
		Nudt18	1.447777742
		E2f6	1.447619499
		Stbd1	1.447444143
		Oas1d	1.447082095
		Lypd3	1.446923079
		Atp2a3	1.446884801
		Nppb	1.446848795
		Gaa	1.446771
		Kns2	1.446697878
		Slc6a12	1.444651421
		Tcfap2c	1.443365015
		Ap2a2	1.44321504
		Slc6a8	1.441391881
		Wnt3a	1.440292279
		2310043N10Rik	1.439678005
		4930455F23Rik	1.439028792
		Lamc2	1.438521567
		Ctgf	1.438196822
		Olfm1	1.437417455
		Ppl	1.436099867
		BC026585	1.435866093
		EG244911	1.435439103
		Fhl1	1.434264933
		Dgat2	1.433006845
		Pts	1.432764067
		Emid1	1.431746639
		Smap2	1.43098229
		2310004N11Rik	1.430854798
		2410076I21Rik	1.430386145
		Trappc2l	1.430108778
		Sohlh1	1.42947624
		Soat1	1.428348456
		LOC638935	1.428290404
		Mpped1	1.428009384
		Sepx1	1.42717769
		Prkaca	1.426043119
		Oat	1.425903381
		Plvap	1.425685654

C vs. K (+LIF)			
Gene Symbol	**FC down-regulated**	**Gene Symbol**	**FC up-regulated**
		Si	1.424485926
		Car12	1.424421404
		Lmna	1.424287444
		Kctd17	1.424005092
		Rab11fip5	1.423192052
		2700050C19Rik	1.423080768
		8030474K03Rik	1.422627555
		Arhgdib	1.421366357
		Acaa2	1.420836189
		Mapkapk2	1.41814695
		Grn	1.418096666
		5031436O03Rik	1.41666134
		Gm817	1.414947034
		Arpc1a	1.414768566
		Tm4sf5	1.414330429
		AW555464	1.412665899
		Stard8	1.409166456
		LOC640972	1.407776487
		Gch1	1.407337273
		Moxd1	1.407288261
		Ccdc3	1.407275452
		Tmem121	1.407187007
		Rab28	1.406244774
		2810452K22Rik	1.405381828
		Lrpap1	1.404067857
		Card10	1.403955468
		Ttyh2	1.403169712
		2310016C08Rik	1.4030598
		Tmem50b	1.40241555
		Smarcd2	1.401850324
		Col17a1	1.401840313

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
Enpp2	3.282067982	Mylpf	3.134486573
Car14	2.585868843	Taf7l	3.113325407
Gdf3	2.579384905	Tgm1	2.965140707
Meis2	2.448402593	Zbtb32	2.868511848
Aph1a	2.324678447	Ddx19b	2.817229609
Phlda2	2.317281544	Fgfr2	2.778122502
Grb10	2.274324383	Gng13	2.734942193
Cxcl12	2.138328107	S100a6	2.728441332
Slc7a3	2.134265209	Laptm5	2.709483131
Mid1ip1	2.010946228	Lgals3	2.473378317
Pycr2	2.003572105	Acta1	2.45118373
Cdc42ep5	1.931969205	Igf2	2.440623862
Hes1	1.921748833	LOC677144	2.426240514
Ccnd2	1.902727363	1110008P14Rik	2.349132049
Epdr1	1.863138361	LOC100047651	2.327532424
Kras	1.845355491	Slc6a8	2.286114354
Rasd2	1.839278041	Taf9b	2.260322888
St6gal1	1.836163262	Mt1	2.191215509

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
Fgf17	1.835248383	Rsph1	2.178901483
Sall2	1.821381533	Hmgn3	2.156405628
Fiz1	1.817686083	Htra1	2.143831735
Gtf2i	1.807496438	Rbp1	2.139827194
Nanog	1.804238684	Gpx2	2.112935678
N6amt2	1.802611787	Stat3	2.103077645
Pde1b	1.801713946	Bgn	2.096784948
Igfbp3	1.776310856	Myl4	2.083909042
Ctgf	1.768490514	1500009L16Rik	2.07855126
ENSMUSG00000074075	1.764390789	Calml4	2.054308719
Pou5f1	1.750005853	Dnmt3l	2.050314926
Rnf130	1.746332525	Wfdc2	2.049863615
Zcwpw1	1.730960538	Tcea3	2.046496551
Tlr2	1.727410819	Atp12a	2.044471716
Elmo1	1.726926541	Psors1c2	2.038413594
Atp10a	1.725579119	Id2	2.031667715
6720469N11Rik	1.723206041	Mfsd7c	2.024522476
Gpr23	1.720477481	Flnc	2.006094223
2610019E17Rik	1.718050328	Prkcb	2.001900518
2310045L10Rik	1.715541377	Krt19	1.964742174
3110013H01Rik	1.714658002	4933421H10Rik	1.961364337
Mid1	1.712865979	Gbp2	1.947587396
Nudt19	1.676641579	2700050C19Rik	1.92057363
Vldlr	1.675673945	Ptrf	1.915848441
LOC381302	1.674856837	Mmp17	1.912763908
Nfatc4	1.672498137	Mfge8	1.894434882
LOC666559	1.670092448	Fbxo2	1.894382691
0610006I08Rik	1.66319372	Plcd1	1.88986597
Phc1	1.66050201	Litaf	1.878136149
Sulf1	1.658905187	Fam102a	1.870870921
Gli2	1.655621135	Rbp7	1.86078853
AI837181	1.64547531	Cxcl16	1.86022879
Cbr3	1.645409118	Gas6	1.846095053
AF067061	1.641851686	Fos	1.838278094
Ecd	1.630105912	Spink2	1.824864459
LOC381844	1.622976945	AU018091	1.820566283
Mycl1	1.621340476	Ckb	1.812496223
Grb7	1.619751322	Cyb5r3	1.799497984
Hist1h3d	1.612600218	Pqlc1	1.794962635
Silg111	1.607960377	Slc5a5	1.794032156
Gm50	1.605888404	Prr13	1.79122658
Fgfbp1	1.596426291	LOC100048733	1.777226825
Hist1h2bf	1.59596316	Mtf2	1.774461266
Foxh1	1.59147807	Pygl	1.767711848
Hist1h2ab	1.587180715	Dusp4	1.766242116
Hist1h2ad	1.585917078	Hbegf	1.766136776
Hist1h4a	1.585763202	Cib2	1.763350882
Hist1h3h	1.575049673	Tex19.2	1.763104291
Pml	1.571352008	Pip4k2c	1.762731794
Hirip3	1.570736512	Klf5	1.755264019
Satb1	1.570436402	Arpc1b	1.750311887
Lass2	1.570159907	Hebp1	1.749642784
Kbtbd2	1.57012987	Spag1	1.746772968
Hist1h2be	1.568744147	Mt2	1.742222693

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
1700013A01Rik	1.56588456	Dkk3	1.739933003
Dis3l	1.564725241	Metrn	1.735734495
Hist1h3e	1.564645529	LOC100045780	1.722695138
2610002J02Rik	1.560889594	Hcn2	1.717330882
Bckdha	1.560302116	Lgals1	1.71550126
Mrpl34	1.558558174	Csrp1	1.714522299
Tcea2	1.558242816	Tcfcp2l1	1.713955395
Hdgf	1.556555749	Rasa3	1.713424476
Abcb8	1.553976162	2810003C17Rik	1.709267054
Trib3	1.552462868	H19	1.701038566
Hist1h2ag	1.550852367	Rell1	1.699810774
Rdm1	1.548917421	Tmem130	1.697483495
Gm428	1.548378715	Slc29a4	1.693159429
Kcnk1	1.548312753	Icam1	1.689666182
Deadc1	1.546792348	Pja2	1.677511834
Hist1h3a	1.545276303	Cryab	1.668857359
Npc1	1.545087932	Srr	1.664943569
Bahcc1	1.543655701	Anxa5	1.662243262
1700034H14Rik	1.543065611	Tns4	1.659072414
Npm3-ps1	1.540814298	Col5a1	1.659003156
Upp1	1.540139077	Hsbp1	1.656822289
Ddx25	1.53901297	Ctnnal1	1.655310305
Hspb6	1.535813065	2700060E02Rik	1.654268662
Ctsc	1.534494209	Gadd45a	1.650566209
Sfrs5	1.529731275	Crlf1	1.648885976
Ilf3	1.527931606	Ephx1	1.645741487
Smo	1.527519724	LOC245128	1.645454276
Creld1	1.527206328	Rgs17	1.645187466
Hist1h2ah	1.524646552	Ccdc19	1.641949704
Mnd1	1.521963424	Klrg2	1.640094326
C330034C07Rik	1.518871148	Col4a2	1.635203457
Mettl3	1.517471659	Gltp	1.628744814
D14Ertd449e	1.517073489	LOC665753	1.628087681
Rhobtb3	1.516496448	Tspo	1.62201011
Hist1h3f	1.515465339	Rab11fip5	1.621979798
Oprs1	1.512215513	Nid2	1.621761687
Nol5a	1.509382328	Ostm1	1.620079253
Hax1	1.508919563	Anxa6	1.619412946
Zfp219	1.508176619	LOC674195	1.615680043
Hist1h2bm	1.506937976	Tceal5	1.615589401
Rrp1b	1.506456349	Lamc2	1.612847048
Aip	1.50611377	Cd9	1.611991988
LOC639910	1.504472664	Ehmt2	1.610938981
Yap1	1.504448019	Nptn	1.609815672
Ubr7	1.50444697	Acss1	1.609438505
Rdh11	1.503493526	Ctsb	1.608995321
Exosc5	1.501482096	Rnase4	1.608952851
Kdelc1	1.500349256	Adam15	1.605162994
Hist1h2bj	1.499083723	Snap29	1.602879256
Prkcbp1	1.498342294	Amn	1.602861428
Thoc7	1.498209962	Cd74	1.599153191
Btbd6	1.496870953	LOC100045280	1.598070087
Med12	1.496458192	Krt17	1.596937485
Hist1h2bh	1.495619079	Plk3	1.593835054

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
Mat2a	1.495547121	1700088E04Rik	1.588133807
Dhps	1.493331556	Hspb1	1.587872978
Mfsd10	1.490936356	Rgs10	1.586600167
Atp10d	1.488950667	Nme5	1.585684557
Xpot	1.486582794	Rab3d	1.584777018
Trip6	1.48626481	Tfpi	1.582386545
1190005F20Rik	1.484825205	Qpct	1.576757571
Hist2h2ab	1.48444581	Cenpf	1.576538772
Nsmaf	1.48413339	Blvrb	1.576463126
2810432D09Rik	1.482653483	Lamb3	1.571841044
Hist2h2be	1.482333265	Aqp3	1.570694293
Yif1b	1.480894042	1700123J19Rik	1.566911283
LOC100045005	1.480838206	Atxn10	1.565099008
Slc27a2	1.480271864	Spag6	1.563932936
Npm3	1.478663938	Clmn	1.562948352
Pus3	1.478485013	Rab28	1.560062886
Pold2	1.47843329	Tmem50b	1.558212266
Bola1	1.476681937	Ap2a2	1.557158939
Ramp2	1.476666607	Slc25a30	1.556376497
Stx3	1.475843594	Aldh4a1	1.556026006
Asxl1	1.473801566	Jak2	1.554799145
Zswim4	1.473665349	Krtdap	1.554311513
Dido1	1.471676824	Capns1	1.553701539
Bcor	1.470494214	Trim71	1.552417388
Tcstv1	1.470058357	Fam129b	1.551531846
Hist1h2bk	1.47002438	Coro1c	1.549127403
2610003J06Rik	1.469447976	Psmb9	1.548791229
Rbpms2	1.468496145	Hist1h2bc	1.548717114
Lrig3	1.468397227	Abcb1b	1.548181301
Mapk12	1.467841042	Rfx2	1.547924109
Bcl11b	1.466837459	Mapt	1.546788971
Rpp25	1.465304576	1500031L02Rik	1.546495298
Sox11	1.464280284	8430427H17Rik	1.545864291
Ier5l	1.463734097	Dgat2	1.543052195
Isy1	1.461636671	Arhgdib	1.540509923
Ssbp1	1.461383279	Tcfap2c	1.537437325
Fgf13	1.461293344	Skap2	1.537241904
Pitx2	1.460783532	8030474K03Rik	1.536171435
Ufc1	1.458228351	Acpl2	1.534318858
Rab34	1.45729513	Impa2	1.530710794
Slitrk5	1.456870283	Rb1	1.530626863
Nrcam	1.454739236	Bmp8b	1.529930611
Rnf145	1.452960797	Lmna	1.527558445
Il4i1	1.45139787	Twsg1	1.527553289
Arid1a	1.448331475	Coro1a	1.527210064
Zfp260	1.447018924	Fhl1	1.527030336
Mrpl3	1.446974941	Ass1	1.526610676
Cct3	1.446803182	3100002J23Rik	1.526101871
LOC627985	1.446129919	2900060B14Rik	1.525695062
Wdr74	1.445533211	Ccnd3	1.525657703
Ppp1r8	1.442617868	Rbm47	1.52435491
9430029K10Rik	1.442549886	Slc30a3	1.523215482
Otx2	1.442098183	Etnk1	1.522768195
Nmral1	1.441542596	Lpp	1.518868336

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
LOC100045738	1.440962398	Fam115c	1.518492385
Dorz1	1.440247013	Pdgfa	1.51837186
Polr1a	1.439218429	Itgb4	1.518062212
LOC381947	1.438965434	Mgst3	1.517723238
Megf8	1.438205775	Ppl	1.516167675
Stk17b	1.43442172	LOC638935	1.515791555
Hist1h2an	1.433312448	Acadvl	1.514767412
Ndrg2	1.433078648	Ptges	1.514420486
Gm129	1.432617586	Usp48	1.514353483
Ccdc77	1.432327287	Actb	1.513736862
Lrrc59	1.431410534	Gstk1	1.512247352
Sfrp2	1.430717864	Ly6a	1.510395008
Zscan4c	1.429576772	Car4	1.509638921
2500002G23Rik	1.429086937	Peg3	1.50904181
Smarce1	1.427857998	1700047I17Rik1	1.508361984
BC017612	1.427669667	Mslnl	1.507325522
Arl4c	1.426939828	Gadd45b	1.507218749
Zfp334	1.426324998	Mvp	1.506459843
AW548124	1.423691648	Slc25a20	1.506427525
Slc6a9	1.422810848	Ppp2r5c	1.506275368
Tlcd1	1.420917122	B2m	1.504816732
LOC208080	1.419559519	Prei4	1.504758945
LOC100045439	1.418988193	Garnl3	1.504555787
Mrps31	1.418776172	Wsb1	1.503480856
Mrpl44	1.41827072	Rab3a	1.502907163
Fancd2	1.416878437	Slc38a5	1.502012019
Dtwd1	1.416807329	Foxj3	1.5012841
Akt3	1.414125853	Ostf1	1.499172995
2810004N23Rik	1.411355626	Cyba	1.498582095
Angel2	1.41110525	Nuak1	1.498340563
Dgkz	1.410600807	Hsp90aa1	1.497594151
Hbp1	1.410297082	Tek	1.497327305
Hist1h3g	1.408524971	Etfa	1.496865831
Seh1l	1.408354443	AA407659	1.495385648
Tcstv3	1.407627545	3110001A13Rik	1.495362363
Ppil3	1.406355364	Rps6ka1	1.495260007
Dci	1.406301618	Rhbdl2	1.494474732
Smad3	1.40540759	Hmgcl	1.490968402
2410081M15Rik	1.403984759	Got1l1	1.490672206
Nme3	1.402219329	Nagk	1.490572082
Nudt1	1.40123492	Nptx2	1.488606483
Shmt2	1.401132892	Hpcal1	1.487429654
Pla2g12a	1.400324497	Unc84b	1.487391029
		Idh1	1.484779301
		Nfu1	1.480401976
		Osbpl6	1.480373287
		Pramef12	1.478140609
		Pop5	1.477580183
		Mapkapk2	1.477495332
		Ptprs	1.477143752
		Ssbp4	1.477136751
		D16H22S680E	1.476559987
		Rtn1	1.475085122
		Susd4	1.474763208

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
		Tax1bp3	1.474688957
		BC022687	1.474479291
		Ehd1	1.474443008
		Plec1	1.47435654
		Gstt3	1.474351272
		Trappc2l	1.4740472
		Slc44a4	1.473898526
		Acaa2	1.471659819
		Tspan14	1.470565186
		Cpm	1.470023589
		5330431N19Rik	1.469610292
		Mbnl2	1.467036104
		Myo1c	1.465652108
		Slc39a11	1.464843958
		Crxos1	1.459860183
		B230114H05Rik	1.459306099
		Cdc5l	1.459091433
		Copz2	1.457656628
		Sap30l	1.457603406
		1700016K19Rik	1.457567191
		Slc39a4	1.454969124
		Arntl	1.453441698
		Vcl	1.453178323
		H2-Bl	1.453058194
		Gpsm1	1.452838174
		Podxl	1.451868156
		Rpl3l	1.451580239
		4930455F23Rik	1.447930311
		2310036D04Rik	1.447572858
		Sdc3	1.446812474
		Nedd4l	1.446461726
		Atp6v1a	1.446341925
		Pqlc3	1.446255585
		Sirt7	1.445072749
		Chst1	1.444243463
		EG212753	1.443329494
		Pdzk1	1.442335002
		Kremen2	1.442120511
		LOC100047863	1.440910121
		D14Ertd668e	1.440822924
		B230343A10Rik	1.44066822
		H2-T10	1.440014267
		Spata6	1.439289433
		Eml1	1.439010145
		Adamtsl4	1.438763725
		Eno2	1.438637744
		BC026585	1.437116762
		Tmem66	1.436142096
		Lrrc34	1.435472898
		Prkcd	1.433800035
		Acot7	1.433543318
		Map1lc3a	1.432784055
		BC008163	1.429639098
		LOC100048622	1.428598532

C vs. K (-LIF)			
Gene	**FC down-regulated**	**Gene**	**FC up-regulated**
		5033414D02Rik	1.428529647
		Aof1	1.428033213
		Mybl2	1.428030536
		LOC381738	1.426583032
		Hap1	1.425783294
		Lrrc59	1.424946176
		Sod2	1.42487191
		Tmem9b	1.424385734
		Wnk1	1.423323419
		Fhod1	1.421365421
		Gstm2	1.420909875
		Ankrd13a	1.420371574
		Micall2	1.41908978
		Plat	1.418950646
		Bbs9	1.417855791
		Edem2	1.416643066
		Tgfbi	1.416513655
		Kif3a	1.415022058
		Lypd2	1.413947264
		Sec14l1	1.413000885
		Arpc1a	1.412830175
		Afap1l1	1.412496006
		Tex261	1.412245602
		5730494M16Rik	1.410733418
		Smarca2	1.41014179
		2310007F21Rik	1.409834516
		Triml1	1.409667402
		Tmed10	1.408543725
		Plac8	1.408288981
		Atp6v0d1	1.407074737
		AI115600	1.406738898
		LOC100044566	1.404937737
		Rshl2a	1.404916396
		Dctn3	1.404413349
		Mns1	1.403785105
		Igfbp4	1.403444178
		Ywhaq	1.402552075
		Mbp	1.402006731
		2310043N10Rik	1.40137956
		Vill	1.401129358
		Rnf185	1.400996484
		Tinagl1	1.400603854

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Socs3	7.173990337	LOC381283	5.824513097
Esrrb	5.688409224	Gbp1	5.685245172
Zfp42	5.675484424	Gbp2	5.442416577
Laptm5	4.455346955	Acta1	5.224517903
Klf4	3.774052414	Pitx2	5.02047154
Sgk1	3.760038497	Enpp2	4.764931614
Emp1	3.736516647	Car4	4.738122787
Cobl	3.724368489	Slc40a1	4.250690501

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Spink3	3.559446007	Fgf8	3.50398467
Fbxo15	3.49740608	Tpm1	3.474495869
Tcfcp2l1	3.447720916	Actc1	3.412718167
Slc11a1	3.386191155	Lrp2	3.356448108
Icam1	3.306745861	Kif1a	3.344846745
2410004A20Rik	3.306320447	Ctgf	3.168996526
Tcl1	3.296507801	Ppp4r4	3.076976942
Fbxo2	3.20288071	Slc30a3	3.053313335
Aqp3	3.178170365	Podxl	2.883998851
Klf2	3.169716403	Gpr23	2.847066506
Lrrc34	3.16444244	Tacstd2	2.826886456
LOC100047200	3.080326786	Gja1	2.802874622
Lama1	3.069749775	Myl9	2.769757239
Manba	3.061633398	scl0003547.1_6	2.72649263
Myl4	3.051547924	Otx2	2.693323811
Eras	3.036302891	Pdlim3	2.689897921
Myst4	3.017839697	Efna5	2.645475708
Cpsf4l	2.988433107	Soat1	2.623301436
Mreg	2.96770054	App	2.601421026
Tbx3	2.950185934	LOC100044190	2.585632451
Nupr1	2.943942386	Ddr1	2.570580178
Tdh	2.941161306	Car14	2.493678263
2200001I15Rik	2.843114983	Zyx	2.484054427
LOC386199	2.836664825	Gp38	2.476065702
Clcnkb	2.835470118	Cxcl12	2.469674217
Clcnkb	2.835470118	Lrpap1	2.434997336
Rfx2	2.77732656	Cxcl16	2.433844026
Ly6g6e	2.754609908	Krt18	2.386707051
LOC100043402	2.69812824	Pou3f1	2.385475248
Jam2	2.68472486	Plekhg2	2.384706787
2310014G06Rik	2.684243002	F830002E14Rik	2.37699608
Calml4	2.673822141	Prkcbp1	2.372193777
Rhox10	2.658383022	Rhobtb3	2.371454182
Spp1	2.647244003	Meis2	2.349974436
LOC270589	2.635844633	St6gal1	2.314651808
Mras	2.629122007	Cst3	2.308943084
Slc29a1	2.62619459	LOC677448	2.293359348
Tuba3a	2.609164734	Psme1	2.265164432
Tex14	2.607286803	Ifi27	2.264312654
Aes	2.577546434	Aph1a	2.261125793
Krt42	2.575632313	Dnmt3b	2.250474279
2410116G06Rik	2.569192892	Ipas	2.247978844
2410146L05Rik	2.568538468	Shroom2	2.243037573
Pfkp	2.563290939	Dok2	2.241204072
1190003J15Rik	2.552946494	Oasl2	2.239274403
Mylpf	2.528013466	Csrp1	2.236436927
Serpinb6c	2.522574508	Cldn3	2.231562824
Mapt	2.503138881	Pdlim7	2.226917975
Gjb3	2.465128979	B2m	2.217971154
1700029P11Rik	2.462076858	Kcnk1	2.216924959
Cdc5l	2.460434699	Cdc42ep5	2.198576889
Rpp25	2.457218476	6720469N11Rik	2.194396383
Aurkc	2.431967242	Dab2	2.191581573
Zfp57	2.425906684	Rab25	2.187508126

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Kdelr3	2.410862462	Atp10a	2.176534176
Acp6	2.367144253	Ccnd2	2.175746532
Timp1	2.352273177	Klf6	2.175661393
Nr5a2	2.349799188	Gpr177	2.173119757
Cxxc6	2.334192258	Plekha1	2.166396172
LOC100044968	2.27702854	Grina	2.16231324
Klf5	2.270847588	Ilk	2.160570991
Kit	2.258826632	Wasf1	2.157460492
Tex19.1	2.252794248	Stx3	2.154518871
Triml1	2.249634702	Bst2	2.141433
Fer1l3	2.24664921	Irs2	2.13173276
Cpn1	2.240148823	Arl4c	2.128069986
Fblim1	2.227769827	Cldn6	2.12089262
Ddc	2.203700098	Lama5	2.115489556
Tcea3	2.190569972	Centd3	2.096669576
1700019N12Rik	2.165801494	Peg3	2.080965177
Prr13	2.149147587	Igfbp3	2.079842528
Upp1	2.145293727	Gbp3	2.048194329
Aard	2.145172709	Prnp	2.037211227
Sod2	2.140341472	Tspan7	2.019654498
Notch4	2.134374611	Gpx8	2.013726432
Skap2	2.12346477	Rbm35a	2.00935115
Liph	2.121933315	Sall2	2.009268428
LOC235857	2.117190979	Kbtbd2	2.003244568
Chrna9	2.11353132	LOC623453	2.002499094
Chrna9	2.11353132	Tmem55a	1.979991612
LOC381844	2.111846417	Myh9	1.979174535
D14Ertd668e	2.110475422	LOC100046120	1.978923837
Dpp4	2.109343269	Epha1	1.972430468
Gpx2	2.09271538	Gtf2i	1.970216129
2410078J06Rik	2.085828486	Rasd2	1.969763594
Tcf15	2.084760692	Ptk7	1.966728177
AU018091	2.074364508	Rab15	1.943904481
Gli2	2.071571322	Rab32	1.943279968
LOC381269	2.070503181	Ghr	1.93795341
Bdh2	2.065057252	Rras	1.934360839
3100002J23Rik	2.056969473	Sox11	1.930515905
Cdyl2	2.055054175	Amotl2	1.929997544
1700123J19Rik	2.053144922	Lass2	1.92636803
C330048F19	2.051534561	Nudt11	1.922929803
2410137M14Rik	2.049880377	Flnb	1.92166412
Ass1	2.045671582	Agrn	1.91758173
Angptl4	2.043068966	Mycl1	1.909593467
LOC100046802	2.039582075	Slc1a3	1.904285108
Etv4	2.036460661	Sepn1	1.9021152
A930010I20Rik	2.035107349	2610528J11Rik	1.901612003
Gm1967	2.031742117	Nfatc4	1.898400051
Ttc29	2.027450871	Stk17b	1.895742152
LOC245128	2.021492656	Cyr61	1.888213146
Calb2	2.016724436	Igf2	1.881485253
Mmrn2	2.016663933	Pawr	1.88141313
Tnfsf12-tnfsf13	2.012499863	Mapk12	1.88046993
Tcfap2c	2.005147454	Grb10	1.878628667
1700061G19Rik	1.985225769	Vasn	1.870425776

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
E130012A19Rik	1.973806713	LOC674135	1.869890769
Ccdc3	1.972290982	Irf2	1.865060673
Pqlc1	1.969218778	Dbndd2	1.864830141
1700012H05Rik	1.956023053	Tpm2	1.863607638
Cd97	1.948872921	Cask	1.861889744
Btbd11	1.94534614	Klf7	1.861340113
Pcsk6	1.942092887	Neu1	1.860751668
Itpka	1.915028786	Krtcap3	1.859025125
Grasp	1.912698986	Rhbdf1	1.858579951
Zfhx2	1.91101526	Polg	1.857380995
Ankrd47	1.909454576	Eif4e3	1.852056273
LOC386298	1.909395236	Gpc1	1.850400175
Gstp2	1.907599756	Plp2	1.8484466
Ptprv	1.9003081	Rgs9bp	1.846608632
Pecam1	1.89909055	2310016C16Rik	1.844719402
Zfp710	1.898834277	D0H4S114	1.844059609
E130014J05Rik	1.890466651	Tmem63a	1.836369385
Zmym3	1.889751211	Hyi	1.836057424
Fcgrt	1.889129296	Rbpms	1.834427601
Slc25a20	1.883425729	LOC100045864	1.833547071
Cyp11a1	1.880017108	9430028L06Rik	1.829825656
Mtf2	1.869086374	Spnb1	1.826434427
Chchd10	1.865173462	Ptpn14	1.82565551
Chchd10	1.865173462	Hexa	1.82552527
Ngfr	1.861611676	Uap1l1	1.824324835
Slc28a1	1.860192826	Gng2	1.817180108
Zfp296	1.859630568	Flna	1.81375352
Gcnt2	1.85431965	Mogat2	1.812911001
Acadm	1.847677996	Trim67	1.801368369
Taf13	1.840726828	Inadl	1.80130972
Pcolce	1.839612464	Fgfr2	1.798725441
Bcl3	1.835573336	Trip6	1.796290975
Ifitm2	1.830803209	Wbp2	1.79623163
E2f1	1.828023706	Cdkn1c	1.79332912
Zfp36l1	1.827396394	Ogfr	1.793274572
Dyrk3	1.826484569	Ctsc	1.792141665
Gm1631	1.823053573	Lmo4	1.791321645
Sox2	1.82021971	Tceal5	1.789373887
Cd9	1.817519048	Kras	1.787093341
Tulp2	1.809114705	AL022832	1.786847609
Lgals3	1.808811643	Cul7	1.784774722
Inpp5d	1.803896929	BC014795	1.784041371
Ampd3	1.801548759	Dock11	1.781283715
Plekha4	1.800681171	Hmgb2l1	1.776698151
Itgb7	1.799717539	Vwa5a	1.774106337
8430410A17Rik	1.797409093	Serpinh1	1.773461885
LOC386330	1.79059632	Endod1	1.77287307
Dnajc6	1.784730029	Fkbp9	1.772084022
BC032203	1.784594349	Pycr2	1.767817016
A130092J06Rik	1.784194245	Prss8	1.766719579
Trim25	1.780456926	Flt1	1.765946635
Gadd45a	1.778203519	Fam171b	1.76574326
Tubb2b	1.77258037	Nrcam	1.765631843
Zscan4c	1.772357645	Plcg2	1.763278729

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
4930504E06Rik	1.768832869	LOC624662	1.760196976
4932425I24Rik	1.768680827	Vldlr	1.75755006
G3bp2	1.765993099	Ssbp3	1.755286546
Gcdh	1.761888133	Pcbp4	1.755207074
Hap1	1.760171408	Rbms1	1.753957225
1500009L16Rik	1.760088581	Tap2	1.75180761
Clgn	1.757135285	Pls3	1.748268472
Clgn	1.757135285	Prickle3	1.747820784
Fez1	1.755711417	H2-K1	1.743725843
LOC673578	1.754862286	LOC100047093	1.742375306
Senp3	1.754847834	2310022B05Rik	1.733135138
BC030476	1.752259108	Flnc	1.729530165
1300013J15Rik	1.747965009	Ppp1r1a	1.729299089
Impa2	1.74586621	H2-D1	1.720275486
LOC208080	1.745748992	Amfr	1.71993449
Ifitm1	1.740394631	1700034H14Rik	1.717905859
0610010I05Rik	1.739494742	Stk39	1.714321264
Prf1	1.732263121	LOC100041569	1.713266321
Stmn2	1.728545488	LOC621823	1.712831776
Ssbp4	1.722382886	Satb1	1.71117963
Tdgf1	1.721142669	Pnpla2	1.71035627
Mt2	1.719472887	1700019E19Rik	1.707928555
Nanog	1.716438984	Tax1bp3	1.704816285
Msh6	1.715589929	Col18a1	1.70163151
Tgfbi	1.7090203	2310045A20Rik	1.70086863
Gdf15	1.705457727	C330034C07Rik	1.700578631
D130003B22Rik	1.705172222	Bri3	1.699984624
Twf2	1.704696807	Mtch1	1.698514892
Aifm2	1.70155858	Acvr2b	1.697671546
Bmp4	1.697222529	Sort1	1.697635867
Mmp11	1.695676442	Rnf130	1.694831215
Hsd17b14	1.691503163	Hspg2	1.692520496
Ulk1	1.691019002	D330001F17Rik	1.691601528
Pdgfa	1.690521191	Tes	1.690267572
Pros1	1.689680542	Npc1	1.690215837
LOC100041835	1.683426971	Arhgef16	1.68915089
Ech1	1.683164287	Mdk	1.688528507
Gtsf1l	1.682809742	Tcf3	1.688409825
Myo1f	1.67921291	Mtap7d1	1.684362697
Igfbp7	1.67134608	Zfp496	1.68362331
LOC100047583	1.667236744	Ttyh3	1.68319984
Srr	1.663601804	Scarf2	1.683056356
9430023L20Rik	1.662457157	Smad3	1.681063842
Nfatc2ip	1.661889009	Tmem184a	1.679959232
D11Wsu47e	1.660077361	Igtp	1.67536135
Mia1	1.657695171	Megf8	1.674471408
Ung	1.657608292	Col2a1	1.671053981
LOC386164	1.655948502	LOC666559	1.669000644
Cenpm	1.655255698	Tpm4	1.668883069
Pla2g10	1.654495871	Dag1	1.667917874
Psma8	1.653407352	Med12	1.667790011
1700013B16Rik	1.653068086	Fgf13	1.667767348
Mcam	1.651837081	Clic6	1.667725775
Dock6	1.649834528	Gns	1.667330546

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Trip12	1.648649106	Rgs17	1.666246883
Cd3eap	1.646387822	Mgst1	1.665162959
Mgmt	1.64241569	Stbd1	1.661220331
Xbp1	1.641417189	Igsf9	1.655599416
1700025K23Rik	1.641097504	Mapkapk2	1.654626737
Aplp1	1.640540918	4631426J05Rik	1.653064846
Stat4	1.639015553	Punc	1.651691555
Tgm1	1.6345663	Cxcl10	1.651624866
LOC638935	1.634515625	Stard10	1.651002149
C330036H15Rik	1.633927166	Gm784	1.648916019
Tekt1	1.633492906	Parva	1.64870951
Pgc	1.63303412	Notch3	1.642993967
Sfrp1	1.631992675	Spire1	1.641575323
Rtn1	1.630214653	Acsl5	1.640277678
Rarg	1.625801689	Sulf1	1.639005846
Amhr2	1.620593242	Nuak1	1.638346223
Syce1	1.618516784	E130102H24Rik	1.636411106
Fbp2	1.615838157	Smo	1.634045438
Phf17	1.614113781	EG630499	1.632583678
LOC100047749	1.613027595	Smarca2	1.631889358
Spire2	1.612701822	Stxbp1	1.631282344
Commd5	1.61113339	Ptprs	1.630875504
2010009J12Rik	1.60968663	Arhgef18	1.630063143
Itpk1	1.607028298	Sox4	1.627869367
Dnmt3l	1.606223217	Rab34	1.627711654
Pcolce2	1.605745217	Il17rd	1.627353439
Cltb	1.605644476	Espn	1.626525987
Nphs1	1.605592586	Snurf	1.625855785
Csad	1.605303289	Arsa	1.623631146
Ssr2	1.604930036	Nr6a1	1.622068602
mtDNA_ND4	1.604650628	Pcdh1	1.62155819
Zbtb7a	1.601347305	Igfbp2	1.619710145
Tmem51	1.598417386	Zfp608	1.619179752
Plxdc1	1.597867861	Igsf1	1.614503046
LOC100045877	1.597492687	Ercc2	1.614261945
Brca2	1.593964132	Ptpn21	1.612607915
LOC100046401	1.593740183	Arid3b	1.607115561
Raet1b	1.591324025	Igf1r	1.605900285
Mfap1b	1.590529655	Tcfap2a	1.605656671
Smpdl3b	1.590494876	BC039210	1.605309796
Nol11	1.589579705	Acsl3	1.604439333
Zap70	1.58849992	Cacnb3	1.604293949
Akap1	1.587205614	Stx7	1.603583641
LOC383491	1.587083967	Cib2	1.601067528
Insl3	1.585324029	2900073G15Rik	1.600874287
2410072D24Rik	1.584597676	Bscl2	1.600482247
Xlr4a	1.579062439	Homer2	1.599681106
Napsa	1.57902468	Plxnb2	1.599232434
41883	1.578592289	Tmprss2	1.597355885
Dcun1d4	1.578380164	Smpd1	1.597000283
Aoc3	1.577563015	LOC381770	1.595519221
Aire	1.576539792	Cln5	1.593675899
LOC100045304	1.576320234	Tspan6	1.592374265
Cdc37l1	1.575283909	Stab1	1.591926717

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Rage	1.575052078	ENSMUSG00000068790	1.589172568
AI850995	1.573602163	Vstm2b	1.588159979
Adam23	1.573356718	Fgd2	1.586875259
Rbbp5	1.571199618	Lhfpl2	1.586293298
Spats1	1.570981568	Creb3	1.585645617
Apobec3	1.567504838	Gnas	1.583817503
Vmn2r-ps14	1.567140771	Tuba1a	1.581702487
Tek	1.566673183	Sidt2	1.581492036
Cmtm7	1.5648587	Reep5	1.581231613
Fbxo6	1.562444949	Prkd2	1.579478005
Zeb1	1.560332566	Dbp	1.578576894
Atmin	1.559687749	LOC100048105	1.57578156
AF067061	1.558501909	Egfr	1.575713888
EG434729	1.557943042	Itm2a	1.574865811
Mlh3	1.556649882	Ano10	1.574059975
E2f2	1.555744784	Elovl1	1.5740326
LOC100046457	1.553520101	Moxd1	1.573320823
Cog7	1.551083416	D630014A15Rik	1.572101564
Hist1h2bc	1.550755302	Tbl1x	1.571730432
Lrrc28	1.550467747	Fmnl2	1.570764334
Ada	1.549850397	Triobp	1.569939487
Capns1	1.549038948	Med14	1.567397081
Eif2s2	1.548816415	Has2	1.566990679
EG382161	1.547062468	Eml1	1.566125544
B4galnt4	1.546915293	Svop	1.565096295
2410076I21Rik	1.545516104	Crlf1	1.564306387
Dppa5	1.544647198	BC023829	1.56398655
Rabif	1.54324127	Wwc2	1.56379727
Htra1	1.543012023	Zfp185	1.563780761
Tmc6	1.542590386	Furin	1.563250064
Hsd3b7	1.540305764	Ap1m2	1.563116073
Mif4gd	1.538924448	Hmga1	1.559584961
LOC277927	1.538756123	Nes	1.558058523
Ndp52	1.537218283	Hes6	1.557341486
Hr	1.534593011	Dennd2a	1.556856942
Plk3	1.533837438	Grip1	1.556270098
LOC433801	1.533562132	Gstm2	1.556031157
Zscan5b	1.532446083	Wbp5	1.552983837
Rusc2	1.532364385	Rap2c	1.551581094
Shmt1	1.529960381	Rnd3	1.549251467
Tfpi	1.529365849	Zkscan17	1.548415895
Srxn1	1.529278993	Fgfbp1	1.548011269
Tuba3b	1.528426738	9930039L23Rik	1.546473051
Pga5	1.528074686	Pde1b	1.546471033
Arhgap30	1.527244097	Card10	1.545817148
Znhit1	1.526270225	Supt6h	1.544612912
Atp6v1a	1.526233054	Cd63	1.543928141
Epb4.9	1.526164382	Sparc	1.543339414
St8sia1	1.52605643	Foxh1	1.54289852
Zfp97	1.525750179	Mllt4	1.541717428
Fzd5	1.525653906	Arhgap29	1.540929131
Atp11b	1.525392047	Tceal8	1.540689267
Dazl	1.525270876	Rnpepl1	1.539266618
Cenpt	1.525043956	Orai3	1.535727132

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Cenpt	1.525043956	Dnd1	1.534638623
Rpl3l	1.524752825	Ets1	1.534421561
Rrp9	1.523719357	Bok	1.533753416
Nid1	1.522544801	AI314180	1.533472532
Map2k3	1.521537932	Trio	1.532262775
D16Ertd472e	1.517515879	Vgll3	1.532051664
Bxdc2	1.51689382	Lbh	1.531821511
Nubp2	1.515097815	AW555464	1.530246758
9530048O09Rik	1.5150804	Tbrg1	1.530208449
Etfb	1.514046168	Sepp1	1.528925788
Rrm2	1.513451415	Tmem132a	1.528903438
Gtf2h1	1.511296683	Myh10	1.527579684
Rmnd5b	1.511082087	Ppp2cb	1.52676015
Slc1a1	1.510109619	Nelf	1.526269416
Rhox5	1.509005925	Pim2	1.526159499
LOC333331	1.508911508	2600010E01Rik	1.525971763
LOC383616	1.508910063	Erdr1	1.524787593
1110008P14Rik	1.508287282	Dgkz	1.524217648
Sdf2l1	1.508024761	5031439A09Rik	1.522912282
Ccnd3	1.507940864	Tapbp	1.521700075
H2afy	1.507693877	Wipf3	1.521371488
Arntl	1.506600812	Mmd	1.521346615
LOC386268	1.50605655	Iap	1.521195406
Vwf	1.504503112	Ypel3	1.519523893
Mybl2	1.503814544	AW548124	1.518285223
LOC381284	1.503803169	Atp10d	1.516535416
Ctnnal1	1.503665157	Vcl	1.513752935
Cyth4	1.502657148	Khk	1.513495919
Myo5c	1.502287864	Stag2	1.512754041
Stra8	1.501340238	Rtn3	1.512560476
Gca	1.50027751	LOC100047800	1.512055109
Jarid1b	1.500095141	Slc5a5	1.510503344
Prdm16	1.499793374	Gusb	1.507403247
AU022252	1.499201068	Ttll4	1.506460593
Papolg	1.496856859	Tmem125	1.506201211
LOC669168	1.494240801	BC057371	1.50515989
6330407J23Rik	1.49359483	Csnk1d	1.504219576
Ptpn6	1.493447095	Zer1	1.50289035
Gprc5a	1.488827076	Anxa2	1.502680247
Akap11	1.488216846	Ube4b	1.501879572
Abcf2	1.487835588	Zfhx3	1.501177193
LOC233184	1.487619067	Dtx4	1.500106037
Mrpl52	1.487041062	Sypl	1.499743774
Tbc1d13	1.485161383	Tcf7	1.498447013
Eif1ad	1.484384724	Wwtr1	1.498153975
Arl6	1.48426705	Chst1	1.497740082
AI467606	1.483328138	7420416P09Rik	1.496925533
Mt1	1.482837456	Igfbp5	1.496866075
Ccdc113	1.481870436	Ndrl	1.496646942
Blvra	1.481372416	1190005I06Rik	1.496640297
Retsat	1.479401315	Gstm5	1.496210293
Stat3	1.479155573	Cad	1.49552514
Tex19.2	1.478419825	Cyba	1.495317375
Foxn4	1.478397426	Scand1	1.495073377

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Bbs2	1.47787911	Gpx3	1.495037719
Igsf21	1.474979514	Rhpn2	1.494807531
Elac2	1.473786177	Nudt19	1.494292
Ak1	1.473696222	Pef1	1.494238733
Nqo2	1.473368588	Bcl11b	1.494204397
Aars	1.472117843	LOC654467	1.493853645
Crtap	1.472093807	Car12	1.491285234
BC019806	1.471679132	Krt19	1.490953817
Ly6g6d	1.471439347	Kctd10	1.490908812
Gm288	1.469142409	Tmem54	1.490739108
Tfrc	1.468910492	Arhgef5	1.489352917
Rasa3	1.468124525	Tpbg	1.488325695
Vegfa	1.467908596	Them2	1.487935814
2810474O19Rik	1.466881714	Akt3	1.487608632
Ppp2r5c	1.464717943	Prickle1	1.487520144
BC004022	1.46453929	A430088H15Rik	1.487301196
Nme7	1.464374261	Rbpms2	1.487048289
Zfp28	1.463724135	Lrrk2	1.486174041
Tnfrsf22	1.462294286	Gats	1.485577698
BC021614	1.461670571	Ptpla	1.485146037
Kcnk5	1.460491912	Gnptg	1.484785735
Ubqln4	1.458981634	St14	1.484308426
C430004E15Rik	1.458425685	C920027I18Rik	1.48415771
Ela2a	1.458174107	Pfkm	1.483929207
Csrnp2	1.458166344	Serf1	1.483689322
Dnajb13	1.45780025	Nt5dc2	1.483437245
Ccne1	1.455817185	Gria3	1.483295599
2700097O09Rik	1.455083051	Prmt2	1.481866411
Tesc	1.455062215	Hist1h2be	1.481360125
Sema4a	1.454953515	Ap3b2	1.480682797
LOC100039786	1.452634298	Swap70	1.480299172
Spryd4	1.452442488	Hsd17b4	1.479767576
1810063B07Rik	1.451569718	6330503C03Rik	1.478205521
Jakmip1	1.450161593	Crym	1.477696543
Cox7a2	1.449830475	Inppl1	1.476977964
LOC676724	1.449626558	Bspry	1.47696021
Atp9a	1.44837237	Tmem106c	1.474164654
C2cd2l	1.448360571	Pkp2	1.472922308
Gpx4	1.448349539	2810022L02Rik	1.472890578
Lace1	1.448327769	H2-Ab1	1.471952278
Scp2	1.448123113	LOC100045542	1.471264511
Tcstv3	1.447342721	Abca3	1.470976929
Cib1	1.445742235	Sfrp2	1.470327127
Cib1	1.445742235	Thsd7a	1.469800981
Robo4	1.443344568	Tnfrsf12a	1.469494338
Kndc1	1.443196903	Dtd1	1.468935186
Ppp1r11	1.442810485	AU040320	1.468630722
Grhl3	1.44253126	Glipr2	1.468064084
C130035G06Rik	1.441006357	Dlg3	1.467317749
Acaa1a	1.4407256	1810049H13Rik	1.466912659
Hs3st3b1	1.440469651	Snx21	1.466512801
Frrs1	1.440052531	LOC224532	1.465356113
Ceacam20	1.439456697	Adk	1.465023919
Ier3ip1	1.439262809	Cugbp2	1.464876483

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Mfap5	1.439161614	Cdc20	1.46486667
Atp2a3	1.437103224	A930004K21Rik	1.464548594
6430527G18Rik	1.435314394	Prox1	1.463907046
Nasp	1.434774176	Irgm1	1.463577376
Mrps12	1.434077287	Mll1	1.462246543
Top1	1.433128632	Clk4	1.462210165
Mns1	1.432075651	Fbxl10	1.461995951
Dbt	1.431005191	Dhps	1.461941294
Ei24	1.430886534	Isyna1	1.461436097
Rhbdl2	1.430405048	Obfc2b	1.461102772
D130017D19Rik	1.430146628	Pcsk9	1.460803065
Sephs2	1.429794801	Rbp1	1.46057793
1700037H04Rik	1.42930727	D10Ertd610e	1.460372212
Ldb1	1.429130414	Ube2e2	1.458691256
1300001I01Rik	1.427459801	Myl6	1.458185572
Rars	1.425965437	Cxadr	1.456516094
BC028528	1.425705143	Casc3	1.454779293
Rabgef1	1.425538105	Aldh5a1	1.454537186
Pgs1	1.425400829	5730525O22Rik	1.454365609
5730419I09Rik	1.424639618	Cthrc1	1.453983609
Nsf	1.424168848	Ctdsp2	1.453001288
Heatr3	1.423829743	Dhx34	1.452448379
Ireb2	1.422912815	Kif1b	1.452271182
Slc25a5	1.422886119	Pdgfb	1.451994232
Mak16	1.42157724	Cpm	1.450917125
Tipin	1.419872322	LOC100046608	1.450863187
Jak3	1.419242919	Nup210	1.450511159
Piwil2	1.415738707	Itm2b	1.449971791
LOC100045280	1.415630413	Cmtm8	1.449760921
Siah1b	1.415354778	Chd7	1.44961062
Atp6a1	1.41526894	Timm50	1.449398815
BC038881	1.415071937	Foxn3	1.448938509
Crtac1	1.414654017	Mfap2	1.447449107
LOC100045983	1.414626781	Cln3	1.447006626
Extl1	1.41451235	Itpr2	1.446524784
Wdr5	1.414399727	Igf2bp3	1.444501841
Zcchc17	1.412915346	Rbbp7	1.443991944
Sp2	1.412235057	Slc15a2	1.443445467
4930461P20Rik	1.410638345	Pfkl	1.443427559
Chpt1	1.410515054	5930412G12Rik	1.440930143
Chpt1	1.410515054	Clic4	1.440825549
Znrd1	1.410410809	Pdlim2	1.440409391
2410081M15Rik	1.409901272	Olfm1	1.438507485
Sox15	1.408754353	Mtmr14	1.438381579
Thumpd3	1.408501327	Hapln4	1.437025683
Mib2	1.408454934	scl0003300.1_40	1.436786406
Timm8a2	1.408282821	Mid1	1.436419323
Mrps31	1.408205348	LOC100045005	1.436244549
Pcyt1b	1.407585161	Srf	1.435824288
Ubtf	1.407029868	3110001A13Rik	1.43571536
Gfod1	1.406603095	Pde10a	1.435573887
Uspl1	1.406396763	Lad1	1.434537581
Coq10b	1.404369488	Pcbd2	1.434436567
LOC385959	1.40344653	Tmem98	1.433888456

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
Sphk2	1.403033287	Pi4k2b	1.432859844
Elovl6	1.402716097	Id1	1.431715347
Sod1	1.40206735	Ndn	1.431340337
Grtp1	1.401946519	Rdh11	1.430515357
EG433923	1.401922024	H2-T10	1.430476354
Robld3	1.401539269	Dlk1	1.430372471
Fignl1	1.401438424	Sdf2	1.429799802
Pafah2	1.401114229	Fzd2	1.428765284
Ddx19b	1.400301732	Arpc5	1.428384024
Gpr133	1.400223044	2310045L10Rik	1.427943598
		Dicer1	1.427905802
		LOC629364	1.427714687
		Wtip	1.427590773
		Efna1	1.427070768
		Yeats2	1.42695623
		Gdap1l1	1.42694567
		Sestd1	1.426011282
		Ryk	1.425750589
		Impact	1.424661241
		Nsmaf	1.423900706
		Gpr89	1.423543274
		Zfand3	1.422697468
		2610524A10Rik	1.422389469
		Pacs1	1.420922547
		Armcx2	1.420813572
		LOC100048295	1.420647108
		Klhdc3	1.420536752
		LOC621824	1.420353371
		Smtn	1.419894974
		H2-T23	1.419400693
		3110004L20Rik	1.419047492
		Gclm	1.418882964
		Renbp	1.418516743
		H19	1.418420007
		Rnf103	1.418250038
		Dyrk1b	1.418213386
		Gpi1	1.417471474
		AI448196	1.416617809
		Cd276	1.416512618
		LOC214575	1.415910946
		Per1	1.415678625
		Figla	1.415659291
		5133401N09Rik	1.41556767
		Nudt7	1.415516504
		Stxbp5	1.415146419
		Gcap27	1.414327607
		scl0002617.1_582	1.413294166
		Gpsm1	1.413199129
		Mrps34	1.412553677
		Atp6v0a1	1.412331328
		Cpe	1.412212445
		Hectd1	1.412002529
		Apaf1	1.411950453
		Samhd1	1.411140765

C+LIF vs. C-LIF			
Gene	**FC down-regulated**	**Gene**	**FC up regulated**
		Fhod1	1.410281422
		Lip1	1.409420933
		LOC545013	1.409041284
		Sqstm1	1.408720728
		D14Ertd449e	1.408643507
		Hist1h2bf	1.408223074
		Itgb5	1.406862087
		Pcgf5	1.406504621
		Prtg	1.406427186
		Tmem9	1.405996517
		Dusp3	1.405393807
		Gnai2	1.405383497
		Rpl13a	1.405229513
		Lpcat4	1.405205456
		Fbxo32	1.405059807
		Pvrl2	1.404407876
		Trabd	1.404118975
		Oxct1	1.404076025
		9530095P18Rik	1.403717219
		Ubr7	1.403396778
		Cdh1	1.403332985
		Rnf11	1.403284746
		H13	1.402998581
		Dsp	1.401866036
		1810037C20Rik	1.40139107
		Ss18	1.400931456
		Cln6	1.400870366
		Pabpc4	1.400818721
		Gtl2	1.400748262

Table 5: List of genes deregulated ≥1.4-fold (adjusted P < 0.05) in Hdac1/2-deleted cells at day 2 (day 0 vs. day 2) and day 3 (day 0 vs. day3)

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Egr1	0.000000	2.95558	Atl3	0.000003	-1.40027
Sct	0.000023	2.48233	Nip7	0.000559	-1.40152
Tex19.2	0.000005	2.4284	Zfx	0.000143	-1.40227
Myl2	0.000003	2.39869	Socs4	0.000752	-1.40249
Hmgn3	0.000001	2.36772	Rpusd4	0.000266	-1.40405
LOC381283	0.000000	2.3599	Rnf113a1	0.000646	-1.40422
Blvrb	0.000021	2.34608	Pus3	0.000835	-1.40642

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Myl4	0.000036	2.34431	Mras	0.000336	-1.40675
Ier3	0.000603	2.33569	BC027231	0.000297	-1.40711
Adssl1	0.000001	2.27478	Rbak	0.000008	-1.40886
3110079O15Rik	0.000005	2.25067	Pnrc2	0.000022	-1.40891
LOC100047651	0.000024	2.2437	Slc35f2	0.001834	-1.40969
LOC100045403	0.000014	2.22745	Ubtf	0.000968	-1.41172
Gch1	0.000003	2.22258	2310008H09Rik	0.000125	-1.41252
Taf7l	0.000039	2.20032	Pon2	0.000586	-1.41336
2900060B14Rik	0.001477	2.12691	Setdb1	0.000043	-1.41551
Mlf1	0.000001	2.12097	Rbmx	0.000702	-1.41606
Slc38a5	0.000000	2.08428	Hsd17b11	0.000761	-1.41609
Serpine2	0.000022	2.05423	Ccdc55	0.001012	-1.41622
Ephx1	0.000017	2.04638	2410016F19Rik	0.000453	-1.4189
Crym	0.000162	2.04595	Ddb1	0.000174	-1.42187
Guca1a	0.000002	2.02793	Rpp25	0.000881	-1.42378
Dusp1	0.000042	2.01996	Eif2b1	0.000987	-1.42421
1190020J12Rik	0.000239	2.00749	2410081M15Rik	0.000030	-1.42753
H2-Bl	0.000013	1.99243	Sall3	0.000027	-1.42796
Ttc9b	0.000176	1.98251	Utp14a	0.001112	-1.42845
Wfdc2	0.000006	1.9825	D230004N01Rik	0.000078	-1.42857
Gstt1	0.000049	1.974	E330016A19Rik	0.000000	-1.43161
EG630499	0.000045	1.97206	5730596K20Rik	0.000007	-1.43258
Camk2n2	0.000102	1.96148	C230055K05Rik	0.000130	-1.4331
Rgs10	0.000398	1.94517	4930584N22Rik	0.000335	-1.4341
Ifngr2	0.000291	1.94303	Apex1	0.000751	-1.43554
Ddx19b	0.000135	1.94185	2310044G17Rik	0.000072	-1.43584
Fos	0.000046	1.93321	2310014H01Rik	0.000382	-1.43602
Gas6	0.000001	1.93015	2410137M14Rik	0.002301	-1.43656
Pdlim4	0.000011	1.9181	Stc2	0.000331	-1.43873
Slc46a3	0.000060	1.91671	Gja1	0.000990	-1.44113
1700007E06Rik	0.000056	1.90726	LOC383491	0.000481	-1.4442
EG546894	0.000008	1.90627	Wdr5	0.000634	-1.44499
Fabp3	0.000109	1.90497	C330016O10Rik	0.000256	-1.44535
Cldn6	0.000000	1.90398	5830411I20	0.000031	-1.44775
Thy1	0.000158	1.89636	D1Pas1	0.000261	-1.44832
6330403K07Rik	0.000009	1.89322	Erh	0.000055	-1.44979
Acss1	0.000043	1.89087	Thtpa	0.000733	-1.45032
2600009P04Rik	0.000003	1.88521	LOC100045887	0.000363	-1.45049
Cplx1	0.000003	1.87874	LOC100046744	0.000761	-1.45306
Crabp2	0.000001	1.87479	2600005C20Rik	0.000901	-1.45599
Rab3d	0.000002	1.86981	LOC232887	0.000388	-1.45761
Acot1	0.000001	1.86865	Rcor2	0.001473	-1.45802
Id1	0.000002	1.86849	Wdr74	0.000980	-1.45822
scl0002540.16	0.000097	1.86572	C430020H24Rik	0.000216	-1.45983
Tspo	0.000003	1.86018	Socs2	0.002268	-1.45989

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Hist1h1c	0.000018	1.85638	Wdr43	0.000528	-1.46047
Psmb9	0.000006	1.84834	2010309J24Rik	0.000597	-1.46286
Bmf	0.000001	1.84765	Rarg	0.000042	-1.46399
Acaa1b	0.001308	1.83113	Epb4.1l4a	0.000435	-1.46553
Mapk13	0.000058	1.82866	Fgd1	0.001062	-1.47034
Pgc	0.000106	1.81926	Cdca5	0.000029	-1.47071
BB287469	0.001727	1.81229	LOC386405	0.000051	-1.47082
LOC381844	0.000048	1.81197	Ars2	0.000002	-1.47198
LOC666185	0.000012	1.81039	6330407J23Rik	0.000007	-1.47363
Nrip3	0.000025	1.8098	Nol8	0.000533	-1.47414
Nptx2	0.000027	1.80348	Myo1f	0.000035	-1.47428
Amn	0.000088	1.80267	Hspd1	0.000588	-1.47591
Idh1	0.000087	1.79957	A630072M18Rik	0.000113	-1.47743
Gpx2	0.000109	1.79866	Exosc4	0.001320	-1.47886
Ly6a	0.000640	1.79803	Zbtb45	0.000525	-1.48007
Ctnnbip1	0.000080	1.78811	9630029G12Rik	0.000066	-1.48028
Gpx3	0.000047	1.78711	Zfp473	0.001045	-1.4813
AA467197	0.000004	1.78643	Etv4	0.000846	-1.48131
Coro1a	0.000001	1.78142	C130032J12Rik	0.000293	-1.48274
Tagln2	0.000183	1.7814	2310057K05Rik	0.001991	-1.4828
Zscan4c	0.001393	1.7765	Rbm4	0.002232	-1.48359
Taf9b	0.000000	1.77318	Patz1	0.000204	-1.48407
Lamb3	0.000014	1.77192	Imp4	0.000007	-1.487
Lefty1	0.000040	1.76572	Ext1	0.000077	-1.49014
1110008P14Rik	0.000147	1.76529	Zfp91	0.000400	-1.50193
Csrp1	0.000177	1.7619	Luc7l	0.001050	-1.50418
LOC546233	0.000009	1.75996	Prpf40a	0.002190	-1.50805
D0H4S114	0.000048	1.75861	Isy1	0.000033	-1.50889
Grina	0.000379	1.74832	1110001A07Rik	0.000094	-1.51113
Rims3	0.000003	1.74561	Hrmt1l2	0.000026	-1.51334
Rnase4	0.000021	1.74551	Gm129	0.000653	-1.51519
Rem2	0.000000	1.74501	Sumo3	0.000288	-1.52002
EG244911	0.000007	1.74335	Ccno	0.000181	-1.52256
Hspb8	0.000001	1.74219	Tmem79	0.000207	-1.5237
S100a1	0.000031	1.74174	Etv5	0.000885	-1.52726
Mip	0.000096	1.74059	scl0001487.1_50	0.000317	-1.53115
Reep5	0.000066	1.74047	Smpdl3b	0.000488	-1.53301
Ppp1r3c	0.000002	1.7392	2610020J05Rik	0.000001	-1.53401
Rgs17	0.000209	1.73482	Ktelc1	0.000297	-1.53811
LOC677144	0.000007	1.73389	Rrp1b	0.000003	-1.54328
1110046J11Rik	0.000001	1.73067	EG627299	0.001526	-1.55131
Krt19	0.000364	1.73062	Timm10	0.001813	-1.55414
4933439C20Rik	0.000007	1.73021	Rn18s	0.000647	-1.5552
Ddit4	0.000156	1.73018	5730543M03Rik	0.001296	-1.56026
Hist1h2bc	0.000181	1.72774	D11Ertd636e	0.000023	-1.56747

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Cox6b2	0.000101	1.72587	Zfp423	0.000055	-1.56914
Alox12b	0.000584	1.72412	Zscan10	0.000729	-1.59246
Wfdc10	0.000003	1.72373	6720463L11Rik	0.000004	-1.60875
Spon2	0.000051	1.72268	LOC100043257	0.001092	-1.60909
Sord	0.000144	1.72205	Frrs1	0.000455	-1.61455
Anxa2	0.000007	1.72028	Ccnd2	0.000154	-1.61628
Casp6	0.000048	1.7198	Zcwpw1	0.000014	-1.63905
Ostm1	0.000057	1.7191	Ppcs	0.001906	-1.64414
A830059I20Rik	0.000099	1.7187	Chac1	0.000052	-1.65138
H2-DMa	0.000073	1.71861	Tut1	0.000298	-1.66339
Lrp10	0.000039	1.71815	Tceal7	0.000372	-1.66677
Hebp1	0.000131	1.71781	2700023E23Rik	0.000222	-1.68363
Pdzk1	0.000000	1.71473	Epb4.9	0.000001	-1.68478
Tdrkh	0.000006	1.70105	8430410A17Rik	0.000166	-1.6892
Klk1	0.000721	1.69943	Fgf17	0.000060	-1.68969
Garnl3	0.000014	1.69921	Cpsf4l	0.000112	-1.69418
1700023M03Rik	0.000016	1.69831	Gadd45g	0.000013	-1.7058
Insl6	0.000681	1.69743	Slc28a1	0.000423	-1.71956
2810003C17Rik	0.000016	1.69668	BC032203	0.000000	-1.72213
Rras	0.000048	1.69647	Dusp27	0.002069	-1.7477
Vegfb	0.001390	1.69613	Tlcd1	0.000057	-1.75906
5133401N09Rik	0.000644	1.69248	Gm1967	0.000007	-1.76543
Rhbdl2	0.000938	1.69186	Slc7a3	0.000124	-1.77219
Trh	0.000211	1.68999	LOC100048330	0.000000	-1.79636
Lrp11	0.000090	1.68993	2810017I02Rik	0.000352	-1.80007
Mmp2	0.000148	1.68927	Gli2	0.000002	-1.80297
1190007F08Rik	0.000928	1.68875	Fiz1	0.000023	-1.80583
LOC385167	0.001520	1.68752	Vegfc	0.000021	-1.82795
Lefty2	0.000580	1.6871	E130014J05Rik	0.002123	-1.84454
Tcfap2c	0.000015	1.67483	Msc	0.000013	-1.89111
1500009L16Rik	0.000755	1.67205	D130003B22Rik	0.000002	-1.93628
Mpnd	0.000167	1.6719	Spink3	0.000284	-1.95023
Hcn2	0.000011	1.67176	Gdf3	0.000007	-1.96818
EG623230	0.000006	1.67132	Senp3	0.000037	-1.97352
Socs3	0.000075	1.66939	Enox1	0.000055	-1.99205
Gp38	0.000104	1.66854	Meis2	0.000020	-2.01398
LOC381727	0.000045	1.66571	LOC100046802	0.000024	-2.01998
Arhgef4	0.000097	1.66504	5033413D16Rik	0.000001	-2.02181
Aldh3a1	0.000426	1.66456	Gbx2	0.000002	-2.12347
Kdelr3	0.000688	1.66217	Setd1b	0.000037	-2.13155
LOC666238	0.000486	1.66181	Hmox1	0.000005	-2.14207
Acpl2	0.000000	1.66049	Zfp428	0.000008	-2.15732
Litaf	0.000018	1.6577	LOC208080	0.000010	-2.18269
Slc39a11	0.000005	1.65723	2610019E17Rik	0.000227	-2.39737
Rbp7	0.000026	1.6531			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Dtnbp1	0.000154	1.65138			
Phox2a	0.000020	1.64644			
Asphd2	0.000720	1.64641			
Sdc3	0.000050	1.64617			
AW212394	0.000005	1.6442			
Dcxr	0.000361	1.6423			
BC061212	0.000128	1.6412			
Mfge8	0.000015	1.64037			
Bik	0.000083	1.64005			
Copz2	0.000056	1.63975			
3110018K01Rik	0.002337	1.63663			
2810410A03Rik	0.000240	1.63662			
Ckmt1	0.000482	1.63621			
LOC100045343	0.000008	1.636			
Rassf5	0.000143	1.63477			
Rsph1	0.000164	1.63308			
Lbh	0.000003	1.62991			
4930583H14Rik	0.001189	1.62951			
Rtn1	0.000018	1.62885			
Ptp4a3	0.001368	1.6272			
5031436O03Rik	0.001354	1.62666			
LOC100044204	0.000013	1.62359			
Txnip	0.000037	1.62268			
Mgst3	0.000553	1.62028			
Apeh	0.000007	1.61943			
Ctsh	0.000012	1.61676			
Rhox10	0.000508	1.61537			
8030474K03Rik	0.000125	1.61517			
Got1l1	0.000075	1.61503			
Gstt3	0.000194	1.61149			
Spink2	0.000001	1.6103			
LOC244061	0.000853	1.60989			
OTTMUSG00000010537	0.000008	1.60826			
Cd74	0.000460	1.60748			
Acta1	0.000255	1.60564			
Ctsb	0.001172	1.60454			
Pygl	0.000203	1.60453			
5031439G07Rik	0.000037	1.60365			
Gnaz	0.000017	1.60351			
Gm1467	0.000106	1.60349			
Carhsp1	0.001587	1.60338			
Tex101	0.000424	1.60205			
LOC266459	0.001913	1.60152			
Id3	0.000168	1.59976			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Pdlim3	0.000040	1.59896			
Mxra7	0.000055	1.59184			
Rhbdl3	0.000091	1.59142			
Sohlh1	0.000008	1.59115			
Commd10	0.001339	1.58987			
Anxa5	0.000085	1.5871			
LOC100045864	0.000088	1.58489			
Fez2	0.001221	1.5843			
Arhgdig	0.000987	1.58204			
Cercam	0.000165	1.57982			
Gnptg	0.000003	1.57855			
Olfm1	0.000005	1.57692			
Gstk1	0.000845	1.57625			
Lgals1	0.002324	1.57462			
Gpc1	0.000008	1.57164			
Fbp2	0.000123	1.5713			
A530057A03Rik	0.000002	1.57119			
Sccpdh	0.000635	1.56993			
Rtn2	0.000501	1.56902			
Rab11fip5	0.000062	1.56786			
Pts	0.000282	1.56625			
Car12	0.000015	1.56411			
Ostf1	0.000042	1.56397			
Ell3	0.000034	1.56395			
Lzts2	0.000120	1.56354			
Ccdc68	0.000068	1.56216			
D9Ertd280e	0.000483	1.56112			
AW555464	0.000256	1.55991			
Cd164l2	0.000006	1.55972			
Crxos1	0.000009	1.55748			
A430089I19Rik	0.000004	1.55702			
Pkp2	0.000381	1.55666			
Col5a1	0.000309	1.55612			
LOC638935	0.000414	1.55516			
1110032E23Rik	0.000070	1.55226			
Cidea	0.000213	1.55172			
Stbd1	0.000078	1.55144			
Triobp	0.000185	1.54967			
Cstb	0.000004	1.54951			
Oat	0.000222	1.54949			
Jak1	0.000038	1.54748			
Tcstv1	0.001628	1.54609			
Dkk3	0.000002	1.54126			
9130213B05Rik	0.000010	1.54113			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Prkra	0.000494	1.54013			
Csrnp2	0.000152	1.53804			
Sertad1	0.001431	1.53783			
Rln1	0.000182	1.53619			
Actn2	0.000002	1.53523			
Gm1673	0.001399	1.53254			
Flywch2	0.000002	1.53177			
Bzrap1	0.000009	1.531			
Susd4	0.000427	1.52895			
BC026585	0.001111	1.52724			
Htra1	0.000977	1.52709			
2410076I21Rik	0.000930	1.52675			
1810020D17Rik	0.000100	1.52584			
Gal	0.000408	1.5252			
Tax1bp3	0.000685	1.52436			
Fgd2	0.000567	1.52432			
Dmrtc2	0.000420	1.52414			
Hist1h4i	0.001750	1.52051			
Chi3l1	0.000308	1.51834			
Wdr92	0.001006	1.5165			
Twsg1	0.000695	1.51481			
Grn	0.000324	1.51458			
Ifi30	0.002299	1.51264			
Capn5	0.000138	1.51169			
Tceal5	0.000124	1.51108			
Nkd2	0.000232	1.51081			
Tes	0.000054	1.50858			
Pard6g	0.000357	1.50824			
2410088K16Rik	0.001654	1.50824			
Defb42	0.000815	1.50794			
Stag3	0.000459	1.50722			
Nrarp	0.000007	1.50644			
3110040M04Rik	0.000221	1.50592			
Zfp36	0.000028	1.50516			
Flot1	0.000109	1.50513			
Dusp26	0.000162	1.50458			
Stxbp1	0.000037	1.50278			
Papss1	0.002052	1.50183			
BC021614	0.000118	1.50087			
Lgi2	0.000213	1.50014			
LOC100044298	0.000065	1.49934			
Rasa3	0.000082	1.49862			
2210408F11Rik	0.000006	1.49818			
Gm2a	0.000784	1.49814			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Casp14	0.000035	1.49742			
Acot7	0.000037	1.49709			
Unc84b	0.000615	1.49497			
Acbd4	0.000160	1.49473			
LOC236311	0.000106	1.49441			
Hspa2	0.000012	1.49423			
Tmem45b	0.000192	1.4941			
Mmd	0.000000	1.494			
Crlf1	0.000298	1.49313			
Prmt2	0.000033	1.49232			
Igf2	0.000167	1.49175			
Tnfaip8	0.001989	1.4901			
EG434729	0.000151	1.48993			
Matn1	0.000645	1.48866			
Tgfb1	0.000883	1.48866			
Tmem130	0.000052	1.48858			
Tuft1	0.000218	1.48823			
Txndc13	0.000001	1.487			
Pacsin1	0.000000	1.48658			
Pip4k2c	0.000160	1.48615			
Pnma2	0.000168	1.48566			
LOC100046207	0.000078	1.48522			
Acss2	0.000027	1.48449			
Gstm5	0.000785	1.48319			
Cxcl14	0.000171	1.48309			
1700052O22Rik	0.001328	1.48185			
Nudt18	0.000208	1.48146			
Hist1h4f	0.001524	1.48104			
BC080695	0.000454	1.47961			
Neurl	0.000222	1.47912			
Pscd3	0.001010	1.47909			
Fbxo10	0.000304	1.47746			
0610011F06Rik	0.000021	1.47722			
1700123J19Rik	0.001879	1.47635			
Unc13b	0.002241	1.47571			
Cd79b	0.000112	1.47486			
Atp12a	0.000075	1.47469			
Rb1	0.000023	1.47264			
H1fx	0.000782	1.47135			
2210410E06Rik	0.000283	1.47088			
LOC100047937	0.000085	1.47035			
2310047A01Rik	0.000089	1.46993			
Lypd2	0.000341	1.46873			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Kns2	0.000054	1.46772			
2810011L19Rik	0.000395	1.46118			
2310040C09Rik	0.000225	1.46108			
Cib2	0.000360	1.46066			
Agap1	0.000118	1.45906			
Alox5ap	0.000678	1.45881			
Plac8	0.000793	1.45857			
Tmem66	0.000015	1.45726			
Krt8	0.001269	1.45706			
2310005E10Rik	0.001542	1.45692			
scl0002507.1_236	0.000203	1.45566			
Ccbl1	0.000284	1.4556			
Rec8	0.001062	1.45444			
Dap	0.001060	1.45249			
Cdkn3	0.000011	1.45236			
Hist1h4m	0.000533	1.45181			
Gpc3	0.000253	1.45124			
LOC100046120	0.002076	1.45082			
Rab28	0.000549	1.45038			
Greb1	0.000142	1.44814			
Rnase1	0.000852	1.44799			
LOC236371	0.001748	1.44728			
Tnni2	0.000681	1.44694			
Gm817	0.002322	1.44675			
Acsl6	0.000008	1.4456			
Apoc1	0.001166	1.44518			
Stard10	0.001219	1.44469			
Mtch1	0.000024	1.44403			
Maged2	0.000120	1.443			
Fam162a	0.001295	1.44275			
Prnp	0.000237	1.44232			
Hist1h3e	0.001202	1.44229			
Hist1h3f	0.001563	1.4408			
Enpp5	0.000347	1.43999			
Phc2	0.001183	1.43933			
Sil1	0.000415	1.43907			
Tuba3a	0.001243	1.43893			
Tmem22	0.000439	1.43862			
Mgst1	0.000470	1.43804			
2700050C19Rik	0.001345	1.43733			
Aldoc	0.000019	1.43721			
Ramp3	0.000589	1.43718			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Acaa2	0.000113	1.43697			
Vps37d	0.000125	1.43675			
Ypel5	0.000071	1.43544			
LOC382183	0.000698	1.4348			
Tpm4	0.001761	1.42637			
LOC100046518	0.000130	1.42636			
Flot2	0.000065	1.42537			
Svop	0.000482	1.4242			
EG212753	0.001816	1.42392			
Nphp4	0.000011	1.4236			
4930539E08Rik	0.000621	1.42355			
D330028D13Rik	0.000623	1.42247			
Tmem159	0.000279	1.42237			
Sirt7	0.001091	1.42231			
Ankrd37	0.000060	1.42186			
Adrb2	0.000793	1.42112			
Etfa	0.000803	1.42052			
2010007H12Rik	0.000009	1.42015			
Aloxe3	0.000343	1.42009			
Gstm6	0.001770	1.41951			
LOC381860	0.000333	1.41904			
9430080K19Rik	0.000562	1.41809			
Alg14	0.000014	1.41806			
LOC100046232	0.000023	1.41749			
Moxd1	0.000026	1.41734			
AI662250	0.000009	1.41697			
Cxcr4	0.000434	1.41566			
Klk13	0.000242	1.41443			
Gltp	0.000969	1.41437			
H2-T23	0.001896	1.41429			
Akr1b8	0.001076	1.41408			
Sstr2	0.000039	1.41355			
Tmem184b	0.000004	1.41293			
Rpl22	0.000070	1.41219			
1700006H02Rik	0.000109	1.40974			
Cldn4	0.000744	1.40932			
Car4	0.000398	1.40886			
App	0.000230	1.4087			
Anxa11	0.000375	1.40826			
Egln3	0.000270	1.40707			
Hist1h4d	0.000672	1.40542			

Day0 vs. Day2					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Prkcb	0.000075	1.4046			
Magee1	0.001549	1.40411			
Tmem53	0.000127	1.40302			
Galk1	0.000031	1.40166			
Pbk	0.000811	1.40162			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Amn	0.000000	6.9085	2410075B13Rik	0.004162	-1.40065
Ly6a	0.000001	5.72	Prrg3	0.003049	-1.40104
Slc38a5	0.000000	5.20997	Tdrd3	0.000069	-1.40184
Ttc9b	0.000001	4.71217	D630036F01Rik	0.000991	-1.40239
LOC666185	0.000000	4.4466	Ide	0.001520	-1.40252
Camk2n2	0.000001	4.44423	Gabpa	0.001498	-1.40283
LOC666238	0.000000	4.39212	Zfp219	0.002830	-1.40303
Tex19.2	0.000000	4.00852	LOC100047226	0.000942	-1.40316
LOC100047651	0.000001	3.94597	Gpr125	0.005035	-1.40343
Hmgn3	0.000000	3.6708	BC027246	0.001889	-1.40352
Taf7l	0.000002	3.54941	LOC626152	0.000195	-1.40406
LOC381283	0.000000	3.40705	D330038O06Rik	0.002360	-1.40472
BB287469	0.000026	3.24502	Gars	0.000497	-1.40532
Tnnc2	0.000064	3.0559	Tbp	0.000482	-1.4054
1110008P14Rik	0.000002	3.01503	Zfp317	0.000810	-1.40562
Blvrb	0.000004	3.00213	B230333E16Rik	0.000061	-1.4057
Hebp1	0.000001	2.96487	Cpsf4l	0.001563	-1.4057
Thy1	0.000005	2.96472	Ilf3	0.001805	-1.40605
Guca1a	0.000000	2.9618	Ahctf1	0.001913	-1.40639
Gpx2	0.000002	2.95862	LOC100047963	0.000780	-1.40644
Cd74	0.000002	2.92514	Auh	0.000234	-1.4067
Cplx1	0.000000	2.92303	Zranb3	0.000000	-1.40691
Crabp2	0.000000	2.88293	EG232875	0.002114	-1.40692
Rsph1	0.000001	2.87801	LOC100042492	0.000937	-1.40753
Cyba	0.000191	2.85587	6820406G21Rik	0.004527	-1.40796
Slc30a2	0.000001	2.84119	Adam23	0.000205	-1.40827
Myl4	0.000010	2.80629	Rnf145	0.000202	-1.40851
Sfn	0.000005	2.74412	Tcea2	0.001031	-1.40879
Id1	0.000000	2.7343	Zfp512	0.002275	-1.40913
Psors1c2	0.000006	2.72188	Mthfd2	0.000809	-1.40921
Htra1	0.000004	2.69736	Smek1	0.000245	-1.40924
Wfdc2	0.000001	2.64864	Ppid	0.001086	-1.40953

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
BC061212	0.000002	2.61955	Tcf20	0.000056	-1.40972
Rtn1	0.000000	2.61695	4930563B10Rik	0.000560	-1.40985
Mt3	0.001455	2.61024	Herpud1	0.000070	-1.41006
Nptx2	0.000001	2.60217	Sirt5	0.000241	-1.41031
LOC546233	0.000000	2.59543	Plekha1	0.004092	-1.41056
Gp38	0.000002	2.56801	9830143E02Rik	0.003307	-1.41093
Reep5	0.000002	2.55158	Gbp1	0.000589	-1.411
Zscan4c	0.000073	2.53872	Hspbap1	0.000810	-1.41254
Sct	0.000020	2.52881	Aprin	0.000193	-1.41277
1190020J12Rik	0.000039	2.52114	Rpap2	0.000005	-1.41328
Tcstv1	0.000015	2.51704	Zfp162	0.001368	-1.41337
LOC100046207	0.000000	2.50923	LOC673501	0.001600	-1.41395
Ela2a	0.000075	2.50005	A430092C21Rik	0.000575	-1.41491
Dusp4	0.000000	2.49642	Tmem47	0.001905	-1.41492
LOC244061	0.000014	2.49343	Bcor	0.000255	-1.41501
2810003C17Rik	0.000000	2.48019	scl0002064.1_2	0.001667	-1.41542
LOC435337	0.000103	2.46832	Map4k3	0.001715	-1.41546
S100a6	0.000034	2.45676	Psma8	0.000641	-1.4157
Rgs10	0.000063	2.44205	D4Wsu132e	0.005877	-1.41581
Crym	0.000041	2.42581	LOC100048372	0.002119	-1.41589
Slc6a13	0.000020	2.42332	Use1	0.000206	-1.41807
Cdkn3	0.000000	2.42044	Mat2a	0.003883	-1.41823
EG630499	0.000008	2.41991	Arl4a	0.000092	-1.4185
AF067061	0.000753	2.41763	Mme	0.000260	-1.41854
Tgfb1	0.000006	2.41536	Myo10	0.000000	-1.41873
Gch1	0.000002	2.41502	Ccdc58	0.000024	-1.41894
Tceal5	0.000001	2.41449	Ccnc	0.000128	-1.4194
Krt14	0.000016	2.41101	Msi2	0.001093	-1.41962
Cldn6	0.000000	2.41004	Aebp2	0.000148	-1.41967
2810410A03Rik	0.000006	2.40958	Ubr1	0.001792	-1.41972
LOC677144	0.000000	2.40016	Gpbp1	0.000612	-1.41987
Id3	0.000003	2.38981	6330407J23Rik	0.000014	-1.42001
1700088E04Rik	0.000001	2.37987	Adprh	0.004029	-1.42005
Crygd	0.000000	2.36094	EG433229	0.002092	-1.42013
Cuta	0.000001	2.35429	Luc7l	0.002456	-1.42042
Cmtm8	0.000087	2.35253	6430590I03Rik	0.000991	-1.42069
Cotl1	0.000056	2.34731	Cla3	0.000058	-1.4208
LOC381844	0.000004	2.34647	Raf1	0.000524	-1.4216
LOC381727	0.000001	2.34203	Ddc	0.000270	-1.42181
LOC266459	0.000053	2.33746	9230108M03Rik	0.001651	-1.42195
2410088K16Rik	0.000019	2.32388	Casp2	0.000054	-1.4222
Fabp3	0.000019	2.31841	Cd2bp2	0.001467	-1.42289

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Vegfb	0.000084	2.31281	Deadc1	0.004812	-1.4231
Gstk1	0.000018	2.30744	BC085271	0.002487	-1.4231
Arhgdig	0.000024	2.30141	LOC100040061	0.000064	-1.42311
Nrip3	0.000003	2.29963	Mbnl1	0.000123	-1.42355
Ctnnbip1	0.000008	2.29635	LOC381285	0.004472	-1.42395
Mylpf	0.000000	2.29251	E030007N04Rik	0.000016	-1.42405
H2-Bl	0.000004	2.28184	Gcnt2	0.000901	-1.42432
Ngfr	0.000001	2.27661	Bccip	0.000312	-1.42451
Acaa1b	0.000212	2.2708	Hnrnph1	0.000352	-1.42471
Tspo	0.000001	2.26846	Prdx3	0.000048	-1.42472
Npw	0.000098	2.26019	Fxr2	0.000009	-1.42479
A430089I19Rik	0.000000	2.25859	A630072M18Rik	0.000208	-1.42483
BC021614	0.000001	2.25704	Papd4	0.001817	-1.42494
Cdk2ap2	0.000154	2.25639	Sntb2	0.000420	-1.42535
Fhod1	0.000010	2.25289	Arih1	0.000257	-1.42543
LOC639910	0.000208	2.24629	Tle4	0.000300	-1.42577
5031439G07Rik	0.000001	2.23548	scl0001118.1_0	0.000409	-1.4258
Adssl1	0.000001	2.23315	Shmt2	0.000433	-1.42768
Mapk13	0.000009	2.2277	9030416H16Rik	0.001181	-1.42798
9130213B05Rik	0.000000	2.22092	Coil	0.000765	-1.4288
Tubb4	0.000000	2.2193	Gmeb2	0.001412	-1.42898
Mlf1	0.000000	2.21487	Dnajc6	0.001623	-1.4291
Hba-a1	0.000482	2.19638	Arid1b	0.000072	-1.42932
Grn	0.000005	2.18958	Cul5	0.000188	-1.42943
LOC236311	0.000001	2.18469	Dbr1	0.000340	-1.42956
Gstt1	0.000020	2.17961	Syde1	0.001150	-1.42986
Sstr2	0.000000	2.17959	Syncrip	0.000039	-1.43047
Ier3	0.001003	2.17681	Mterf	0.000073	-1.43086
LOC331259	0.000043	2.17534	LOC209281	0.005851	-1.43132
LOC245892	0.000195	2.17498	Tnrc6a	0.004693	-1.43135
LOC382183	0.000006	2.15922	Etv4	0.001399	-1.43346
Tax1bp3	0.000015	2.15801	Alkbh	0.000086	-1.43417
Hcn2	0.000001	2.15787	LOC100045567	0.000081	-1.43444
Crip2	0.000009	2.15597	Cfp	0.001068	-1.43465
Rbp1	0.000084	2.1521	Ndufaf1	0.001188	-1.43467
Lrp10	0.000004	2.14319	Sdccag1	0.003841	-1.4364
Tes	0.000001	2.13799	Akap9	0.000003	-1.43645
Ldoc1	0.000000	2.1237	1500012F01Rik	0.000130	-1.43766
6330403K07Rik	0.000003	2.12303	C330048F19	0.002912	-1.43771
Coro1a	0.000000	2.12149	Usp7	0.001252	-1.438
Rap2ip	0.000001	2.11887	Sbno1	0.000125	-1.43805
Myl2	0.000008	2.11871	Trps1	0.000082	-1.43851

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
2900060B14Rik	0.001545	2.11411	Rabggtb	0.000336	-1.43858
Acss1	0.000015	2.11366	Mak16	0.000098	-1.43866
Gm1673	0.000045	2.11291	Gsta3	0.000059	-1.4389
LOC100045403	0.000022	2.11082	Ggnbp2	0.000618	-1.43894
Grina	0.000062	2.10847	Dhps	0.001779	-1.43916
Tmem45b	0.000003	2.10545	Srfbp1	0.003897	-1.43938
Cxcl14	0.000003	2.10434	Rccd1	0.000259	-1.43952
Sdc3	0.000004	2.10128	Tdrd12	0.001585	-1.43991
Ccdc23	0.000307	2.09995	Nol1	0.000015	-1.44029
Gas6	0.000001	2.09655	2210015K02Rik	0.000493	-1.44043
BC030476	0.004676	2.09316	Rnu65	0.001831	-1.44081
Batf3	0.000072	2.09063	Ccnd1	0.000936	-1.44229
Ehd1	0.000068	2.08875	Papola	0.000266	-1.44285
Josd2	0.000423	2.08673	Pak2	0.001942	-1.44304
LOC384298	0.000002	2.0859	Plod2	0.000190	-1.4436
Tspan17	0.000316	2.0834	Cep57	0.000961	-1.44362
Mip	0.000015	2.08331	BC088983	0.000119	-1.44425
LOC100046120	0.000033	2.08166	Eif5	0.002407	-1.44445
EG244911	0.000001	2.07872	Irf9	0.000051	-1.44454
8030474K03Rik	0.000008	2.07563	Slc35f2	0.001243	-1.44464
Fbxo2	0.000778	2.07417	C920004C08Rik	0.000107	-1.44478
Slc39a11	0.000000	2.0629	Cept1	0.000360	-1.44496
Isyna1	0.000116	2.06211	2310007G05Rik	0.000388	-1.44524
1700016K19Rik	0.000007	2.0609	LOC100045005	0.000644	-1.44549
OTTMUSG00000010438	0.000000	2.05423	Krr1	0.001026	-1.44572
Lgals1	0.000152	2.05367	2700092H06Rik	0.000069	-1.44579
Sqstm1	0.000084	2.05221	4631405K08Rik	0.000014	-1.4459
Sccpdh	0.000034	2.05154	Sema4a	0.000352	-1.44721
AW212394	0.000000	2.0513	OTTMUSG00000010673	0.000460	-1.44722
Zyx	0.000007	2.05033	Epdr1	0.000567	-1.44729
LOC386085	0.001854	2.04527	Adal	0.000491	-1.44731
Fam171a2	0.001345	2.03446	Mrpl1	0.000754	-1.44825
Gstt3	0.000015	2.03362	Gtf2i	0.001578	-1.44865
Phf13	0.000000	2.03129	AI314180	0.001660	-1.44876
5133401N09Rik	0.000104	2.02968	Tmpo	0.000275	-1.44889
Asphd2	0.000083	2.02919	Serinc1	0.001475	-1.44892
LOC383616	0.000030	2.02872	Dars	0.000385	-1.45037
Igf2	0.000004	2.02572	Ascc3l1	0.001159	-1.45055
Pmp22	0.000060	2.02538	LOC384382	0.002558	-1.45077
LOC100041290	0.000050	2.01614	Pum2	0.005868	-1.45093
Magee1	0.000018	2.00977	Tmc7	0.002445	-1.45116
Rnase1	0.000016	2.00757	LOC100046035	0.001312	-1.45166

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Spsb2	0.000002	2.00247	C330016O10Rik	0.000238	-1.45167
Rab3d	0.000001	2.0013	2410017P07Rik	0.000386	-1.45245
LOC100046518	0.000002	1.99609	Nphs1	0.000003	-1.45276
Sec11c	0.000388	1.99289	Nthl1	0.000133	-1.45285
Rassf5	0.000016	1.99287	Pon2	0.000369	-1.45295
Rhox10	0.000053	1.99046	A930013G23Rik	0.000084	-1.45296
Stard10	0.000025	1.99041	Rbbp9	0.000329	-1.45319
Lbh	0.000000	1.98987	D11Ertd636e	0.000077	-1.45363
Slc5a5	0.000000	1.98859	Rabep1	0.000213	-1.4538
Maged2	0.000002	1.98835	BC027231	0.000169	-1.45384
Rhox9	0.000178	1.98654	Sbds	0.000106	-1.4539
H47	0.000012	1.98389	Wdr74	0.001021	-1.45438
Dtnbp1	0.000021	1.98344	Fzd7	0.000824	-1.45502
AU018829	0.000000	1.98271	Scml2	0.000235	-1.45591
Spnb3	0.000034	1.97368	C330024D21Rik	0.000369	-1.45595
Tmem54	0.000027	1.97298	EG435970	0.000401	-1.45616
Slc46a3	0.000046	1.97177	6720467C03Rik	0.000031	-1.45678
LOC385167	0.000328	1.97134	Bxdc2	0.000142	-1.45701
Rem2	0.000000	1.96963	LOC100046586	0.001461	-1.45703
Sertad1	0.000091	1.96952	Tlcd1	0.000705	-1.4573
Card10	0.000037	1.96839	Hnrpl	0.005558	-1.45769
EG434729	0.000005	1.96816	Esrrb	0.004961	-1.45805
LOC665290	0.000022	1.96471	LOC100047674	0.000070	-1.45823
Rgs17	0.000058	1.96266	LOC386405	0.000059	-1.45847
Unc84b	0.000024	1.96229	Nanos1	0.000534	-1.45855
Nans	0.000129	1.96131	Ankrd13c	0.000485	-1.4592
Vat1	0.004348	1.95828	Nup62	0.000322	-1.46009
S100a1	0.000009	1.95819	LOC100046393	0.000287	-1.4608
Cd79b	0.000003	1.95735	Tor1aip1	0.000005	-1.46089
Akr1b8	0.000017	1.957	Pank1	0.001669	-1.46099
Klrg2	0.000011	1.95575	Zfp644	0.004716	-1.46124
Ckb	0.000127	1.95495	C030048B08Rik	0.002560	-1.46141
Nagk	0.000227	1.95376	5830411I20	0.000026	-1.46164
Ifi30	0.000133	1.95165	LOC226486	0.000474	-1.46165
AW555464	0.000019	1.94768	Gtpbp10	0.000900	-1.46338
Acta1	0.000029	1.94695	Rnaseh2b	0.002907	-1.46345
LOC100045864	0.000008	1.94021	E330011I20Rik	0.001358	-1.46357
H2-DMa	0.000020	1.93844	2310001H12Rik	0.000012	-1.46469
Serpine2	0.000039	1.93701	Wdr21	0.001109	-1.46494
Mgst3	0.000078	1.93698	Xpo4	0.000003	-1.4654
Hsbp1	0.000056	1.93594	Papolg	0.001075	-1.46612
Rbp7	0.000004	1.93541	Slc35d2	0.003869	-1.46706

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Tcstv3	0.000702	1.9354	Car11	0.002837	-1.46731
Casp6	0.000013	1.93183	Nampt	0.002657	-1.46823
Fdps	0.000073	1.92997	Zbtb26	0.000726	-1.46853
Spink2	0.000000	1.92724	Ivns1abp	0.003008	-1.46871
Mapre2	0.000098	1.92635	Cetn3	0.000623	-1.46872
Ifngr2	0.000321	1.92212	Cct6a	0.001270	-1.46921
Pglyrp1	0.000213	1.92196	Lrrc34	0.000318	-1.46922
Pip4k2c	0.000006	1.92129	LOC100047264	0.000020	-1.4694
Tdrkh	0.000002	1.92073	2310037I24Rik	0.001045	-1.46959
Slc25a10	0.000163	1.92071	Aars	0.000239	-1.46964
Dgat2	0.000625	1.91952	Gtpbp4	0.001851	-1.47012
Tppp3	0.000086	1.91947	Rbak	0.000004	-1.47118
1190003J15Rik	0.001583	1.91129	Ganab	0.000093	-1.4712
Acot7	0.000002	1.91127	Gnl3	0.002951	-1.47143
Gpx3	0.000024	1.90716	Ireb2	0.001912	-1.47172
Slc9a3r2	0.000004	1.9047	LOC100048295	0.000085	-1.47224
2200001I15Rik	0.000667	1.89717	Rragb	0.000266	-1.47245
Tmem120a	0.000203	1.89655	Sall1	0.000231	-1.47275
Prmt2	0.000001	1.89472	Eif4enif1	0.000627	-1.47302
Chka	0.000828	1.8933	Mkln1	0.001101	-1.47323
Phc2	0.000037	1.89295	Hrmt1l2	0.000040	-1.47366
Sirt7	0.000027	1.89129	LOC245350	0.000599	-1.47391
Actn2	0.000000	1.8905	Pcf11	0.000022	-1.47444
Med10	0.003227	1.89017	2810017I02Rik	0.003755	-1.47455
Psmb9	0.000005	1.89013	Ddx26	0.000369	-1.47564
Rims3	0.000001	1.88998	A430106B04Rik	0.002327	-1.47566
Egr1	0.000003	1.88955	Efr3a	0.004051	-1.4763
EG546894	0.000009	1.8895	E030026I10Rik	0.000002	-1.47651
D16H22S680E	0.000000	1.8879	Dhx35	0.000317	-1.47701
Asprv1	0.000019	1.8879	C920006O11Rik	0.000173	-1.47712
Carhsp1	0.000275	1.88738	Cenpi	0.002539	-1.47766
Suds3	0.000000	1.88664	LOC676748	0.001038	-1.47837
Dmkn	0.000032	1.88564	Top1	0.004880	-1.47837
Cstb	0.000000	1.88205	Oxnad1	0.000066	-1.47882
2600009P04Rik	0.000004	1.88185	Gm1815	0.000432	-1.4794
1190007F08Rik	0.000307	1.87816	1200015N20Rik	0.000045	-1.47984
Sh3bgrl3	0.000915	1.87608	Bbs2	0.004321	-1.48003
Crxos1	0.000001	1.87468	Zfp518b	0.000062	-1.48012
LOC212386	0.000330	1.86841	Hspb6	0.000938	-1.4803
4921521F21Rik	0.000002	1.86801	1700081H05Rik	0.000201	-1.48078
Sep-08	0.000098	1.86725	AW549877	0.000683	-1.48153
Rhbdf1	0.000023	1.86649	LOC381302	0.002313	-1.48168

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Stbd1	0.000008	1.86643	Rnf113a2	0.000342	-1.48227
Rab11fip5	0.000007	1.86542	Ccnt2	0.000202	-1.48232
Slc29a1	0.000002	1.86429	Gtf2e1	0.000479	-1.48347
Flnc	0.000088	1.86353	Kat2a	0.000414	-1.48361
Lamb3	0.000008	1.8623	Tanc1	0.002491	-1.4844
Anxa5	0.000012	1.86184	Myst4	0.000158	-1.48471
Col5a1	0.000036	1.86041	Sfrs1	0.000436	-1.48494
Chst1	0.000005	1.85581	Zfp508	0.000905	-1.48506
Map1lc3a	0.000096	1.85451	Pou5f1	0.001378	-1.48521
Rsad2	0.000004	1.85432	Ttc39b	0.000258	-1.48554
Limk1	0.000365	1.85175	Smug1	0.000022	-1.48615
Agpat2	0.000003	1.85013	Tbl1xr1	0.000043	-1.48622
Lypla2	0.000016	1.84932	C130032J12Rik	0.000282	-1.48637
Csrp1	0.000106	1.8483	Ccnl	0.002028	-1.48679
LOC382184	0.000002	1.8471	Tcerg1	0.000350	-1.48703
Rogdi	0.000743	1.84709	3632413B07Rik	0.001582	-1.48754
AI836003	0.000000	1.84648	Fbxl11	0.003498	-1.48764
Tesc	0.000582	1.84586	5730601F06Rik	0.000091	-1.48766
Anxa2	0.000003	1.84341	Prpf4	0.000586	-1.48875
Atp12a	0.000004	1.84265	2410078J06Rik	0.000449	-1.48875
Sohlh1	0.000001	1.84064	2410002O22Rik	0.004916	-1.48949
Ddx19b	0.000229	1.83989	Arid1a	0.000484	-1.4902
C3	0.000054	1.83944	BC038822	0.001803	-1.49026
LOC100040016	0.000091	1.83923	Pprc1	0.000890	-1.49091
Dctn3	0.000502	1.83867	2310008I22Rik	0.001508	-1.49099
Tmem9b	0.000000	1.83728	Ddx52	0.001424	-1.49146
Apeh	0.000001	1.83598	BC020002	0.002834	-1.4932
Tcf19	0.000093	1.8347	LOC544988	0.000550	-1.49378
Klhl21	0.000192	1.83453	2310031L18Rik	0.000578	-1.49444
Sdcbp2	0.000001	1.83342	Inpp5d	0.001790	-1.49555
9130211I03Rik	0.000102	1.83338	Noc3l	0.002218	-1.4966
LOC630179	0.000007	1.83292	Alkbh3	0.000290	-1.4971
Cldn10	0.000002	1.82632	Prmt8	0.000086	-1.49772
Lin7b	0.000014	1.82392	Secisbp2	0.000748	-1.49776
Copz2	0.000016	1.82274	9530048O09Rik	0.000007	-1.49794
Prf1	0.001998	1.8212	Ech1	0.001238	-1.50026
Rhbdl3	0.000017	1.81947	Jtb	0.000033	-1.50159
Nppb	0.000437	1.81919	Nudt5	0.000006	-1.50161
Crlf1	0.000023	1.8191	Chrna9	0.000158	-1.50216
Dusp26	0.000014	1.81821	Oxsr1	0.000419	-1.50276
Phox2a	0.000006	1.81768	Mbtd1	0.002766	-1.50395
Plekhf1	0.000025	1.81734	Pde1b	0.000662	-1.50419

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Fbxo10	0.000020	1.81717	Wwp1	0.001158	-1.50437
EG212753	0.000076	1.81665	Mre11a	0.000419	-1.50441
Pgd	0.000017	1.81509	1810026B05Rik	0.002033	-1.50503
1110019N10Rik	0.000346	1.81395	Slc19a2	0.002853	-1.50612
LOC236220	0.000245	1.81374	LOC234360	0.000057	-1.50617
4933421H10Rik	0.000013	1.81158	Tmem39a	0.000016	-1.50634
Itsn1	0.000144	1.81156	2310005L22Rik	0.005366	-1.50669
Rala	0.000035	1.81009	C530038F07Rik	0.001689	-1.5068
Mapkapk2	0.000170	1.80965	Dbf4	0.003228	-1.50698
LOC100040479	0.000740	1.8094	LOC232887	0.000229	-1.50772
Ctsh	0.000003	1.80905	Sesn2	0.000808	-1.50794
Gnptg	0.000000	1.80675	Cobl	0.000028	-1.5085
Arpc1b	0.000341	1.80665	Irf2bp2	0.000008	-1.50906
Impdh1	0.000527	1.80629	Eif2b5	0.000003	-1.50912
2310040C09Rik	0.000013	1.8042	Mrps31	0.000047	-1.50978
4930519N16Rik	0.000247	1.8031	1110059G02Rik	0.000894	-1.51027
Tmem121	0.001010	1.80267	C130085G02Rik	0.004587	-1.51074
Fcho1	0.000401	1.8015	BC034076	0.001619	-1.51089
Mfge8	0.000005	1.80024	Asxl1	0.000046	-1.51113
Tex101	0.000108	1.79917	Catsper2	0.000042	-1.51311
OTTMUSG00000010537	0.000002	1.79779	Gart	0.002002	-1.51444
Rabl4	0.000276	1.79726	E330018D03Rik	0.000588	-1.51475
LOC384964	0.000144	1.79726	Qtrtd1	0.001961	-1.51482
Alg14	0.000000	1.7967	Noc2l	0.000011	-1.51492
Myl9	0.001285	1.79555	Zfp42	0.000656	-1.51506
Rhox6	0.000032	1.79394	1300006C19Rik	0.003900	-1.51551
Tagln2	0.000170	1.79337	Ddb1	0.000060	-1.51574
Ostf1	0.000007	1.79218	Bcat2	0.000931	-1.51635
LOC100048733	0.002193	1.79143	Atf5	0.000870	-1.51821
Gpc1	0.000001	1.79035	Fkbp10	0.002596	-1.51824
Slc24a6	0.000264	1.78917	C230055K05Rik	0.000050	-1.51846
Psap	0.000463	1.78864	A930010I20Rik	0.000494	-1.51854
Dnmt3l	0.000009	1.78856	1810030N24Rik	0.000014	-1.51925
LOC625360	0.001335	1.78726	Uck2	0.000008	-1.51946
Tmem192	0.000156	1.78567	Dnajb6	0.000006	-1.51969
Ctgf	0.000006	1.78455	C130072A16Rik	0.000778	-1.5203
AF067063	0.000155	1.78384	Hmgb2l1	0.000448	-1.52046
Lypd2	0.000025	1.78338	Hirip3	0.002268	-1.52048
EG623230	0.000003	1.7831	Dtwd1	0.005082	-1.52074
D330028D13Rik	0.000028	1.78248	Ccne2	0.000620	-1.5209
Alad	0.000065	1.78137	9430088P09Rik	0.002575	-1.52204
Rasa3	0.000008	1.77923	Socs4	0.000202	-1.52209

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
H2-D1	0.000241	1.77857	Utp14a	0.000415	-1.52382
9530058B02Rik	0.000105	1.7772	LOC270491	0.001635	-1.52505
Fez2	0.000326	1.77675	Sall3	0.000009	-1.52582
Ints1	0.000249	1.7733	9430081H08Rik	0.002109	-1.52693
Gstp1	0.000537	1.77052	Uspl1	0.000155	-1.52702
Eif1b	0.001590	1.76812	Fktn	0.001077	-1.52813
Adfp	0.000517	1.76797	Atf4	0.002578	-1.52835
Pdlim4	0.000026	1.7676	D030034I04Rik	0.000111	-1.52948
3110040M04Rik	0.000027	1.76522	Clk4	0.000010	-1.53048
B230343A10Rik	0.000000	1.76469	LOC100040353	0.000165	-1.53054
Commd9	0.000010	1.76196	Ctage5	0.003809	-1.53059
Pitpna	0.000122	1.7607	6430527G18Rik	0.003016	-1.53118
Nudt22	0.000109	1.76	Gpt2	0.000004	-1.53127
B2m	0.000001	1.75833	Boll	0.000139	-1.53174
LOC277049	0.000570	1.75808	A830080D01Rik	0.000585	-1.53328
Gsto1	0.000113	1.75615	Gemin4	0.000425	-1.53396
Als2	0.000001	1.75508	Gm525	0.000007	-1.53456
Smcx	0.000185	1.75439	scl0004020.1_31	0.003949	-1.53504
Acaa2	0.000007	1.74733	Cul1	0.002690	-1.53624
Gchfr	0.000096	1.74531	Ccdc47	0.000007	-1.53718
Hyi	0.001028	1.74132	Eif4b	0.000494	-1.53781
2200002D01Rik	0.002258	1.74122	Pias2	0.000008	-1.53829
LOC195150	0.000002	1.73989	Rock1	0.004973	-1.53881
LOC270344	0.001749	1.73819	1200014J11Rik	0.000363	-1.53891
5031436O03Rik	0.000652	1.73621	Ptch1	0.000075	-1.53919
Accn2	0.000156	1.7342	Orc5l	0.000404	-1.54039
Idh1	0.000133	1.73238	Epc1	0.000011	-1.54087
Gnaz	0.000006	1.73219	Rrm1	0.002346	-1.54216
Rwdd2	0.000527	1.72986	LOC548597	0.001587	-1.54225
Phlda1	0.001491	1.72968	Gtf2h4	0.000151	-1.54277
Rnaset2b	0.000085	1.72866	Gbl	0.000013	-1.54287
AW120700	0.000038	1.7283	Dtwd2	0.002014	-1.54299
Mov10l1	0.001381	1.72704	Fgd1	0.000531	-1.54305
Rasl11a	0.000027	1.72698	Fanci	0.005164	-1.54338
1110018J23Rik	0.000093	1.72674	B230363H02Rik	0.001880	-1.54342
1700007E06Rik	0.000165	1.72572	Zfp281	0.000821	-1.54365
Creld2	0.000004	1.7256	Jmjd1a	0.000051	-1.54391
Ccl27	0.000168	1.72441	Dscr1l2	0.000155	-1.54437
Aldh3a1	0.000286	1.72287	2010309E21Rik	0.000252	-1.54446
Srr	0.001472	1.72248	Rarg	0.000018	-1.54513
Akr7a5	0.001595	1.72208	LOC271505	0.004010	-1.54528
Bex2	0.000054	1.7214	LOC100045887	0.000137	-1.54561

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Actc1	0.000000	1.72082	Zcchc3	0.000174	-1.54607
Slc9a3r1	0.000009	1.72077	BC049349	0.001208	-1.54647
Garnl3	0.000012	1.72071	2810008M24Rik	0.000021	-1.54672
1110021J02Rik	0.000624	1.72019	Alpk3	0.000045	-1.54679
Cyb561	0.000005	1.71875	Zfp91-cntf	0.000051	-1.54693
1700019E19Rik	0.000532	1.71866	Anp32a	0.000069	-1.54796
Pdia6	0.000506	1.71862	Hsd3b7	0.000015	-1.54802
Ebp	0.000154	1.71569	Actn3	0.000078	-1.54921
2510002J07Rik	0.000273	1.71381	D930048N14Rik	0.000001	-1.54953
Mrps6	0.000002	1.71321	Hsd17b11	0.000184	-1.55169
Rps6kl1	0.000022	1.71017	2310045L10Rik	0.000191	-1.5519
Gal	0.000092	1.70949	9130422G05Rik	0.001101	-1.55246
Atp6v0e	0.000009	1.70918	LOC624198	0.002279	-1.55248
Camk2b	0.001030	1.70873	1110004P21Rik	0.003449	-1.55251
Lgi2	0.000036	1.70844	Dpp4	0.000004	-1.55313
1110014O20Rik	0.000019	1.70831	A730098D12Rik	0.000412	-1.55363
Rabac1	0.002523	1.70799	Igf2bp3	0.000041	-1.55397
Syp	0.000130	1.70752	Ei24	0.000090	-1.55419
Ada	0.000263	1.7062	Nhlrc2	0.000308	-1.55451
Rhbdl2	0.000858	1.70564	Pml	0.000079	-1.55559
Chaf1b	0.000046	1.70411	Il11ra1	0.000024	-1.55572
Nrgn	0.000231	1.7026	Pim3	0.004912	-1.55644
Mpped1	0.000028	1.70197	LOC433721	0.000597	-1.55673
Mapk8ip1	0.000001	1.70174	Zfp91	0.000237	-1.5573
Myh3	0.000040	1.70103	2610524F24Rik	0.000082	-1.55808
Rfxap	0.000456	1.70098	Eno3	0.001635	-1.56008
Reep6	0.000365	1.69951	LOC333751	0.003911	-1.56018
Slc35c1	0.000003	1.6981	Fez1	0.000440	-1.56041
Kcnh3	0.000227	1.69763	Sep-02	0.000547	-1.56062
Zfp36	0.000005	1.69713	2310014H01Rik	0.000105	-1.56072
Dbndd2	0.000065	1.69421	Smg7	0.000291	-1.56105
Bmf	0.000004	1.69374	Trim2	0.000176	-1.56163
Morn2	0.000175	1.69347	Lypla1	0.001535	-1.56214
2810402K13Rik	0.000061	1.69346	Rnf113a1	0.000120	-1.56284
Olfm1	0.000002	1.69335	Psip1	0.000945	-1.56393
4930502E18Rik	0.000011	1.69321	4833442J19Rik	0.000128	-1.56512
Pdlim3	0.000019	1.69255	Zfp451	0.000583	-1.56559
Emilin2	0.000365	1.69202	Atl3	0.000000	-1.56633
Tpd52l1	0.000054	1.69047	Ext1	0.000035	-1.56805
Tmem22	0.000044	1.68955	Zfp292	0.000553	-1.56953
Prkra	0.000150	1.68933	0610006I08Rik	0.000283	-1.56977
Spon2	0.000065	1.68901	Fxr1h	0.000313	-1.56983

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Cercam	0.000070	1.6869	Asz1	0.000039	-1.57035
Ptrh1	0.001342	1.68607	3110009E18Rik	0.000213	-1.57063
Tmbim6	0.000829	1.686	Pafah1b1	0.000155	-1.57105
Elovl4	0.000192	1.68595	Ufc1	0.003727	-1.57177
Gprasp2	0.002584	1.68436	Gtdc1	0.002069	-1.57183
Mlycd	0.005281	1.68313	Marveld1	0.000095	-1.57204
C2cd2l	0.000229	1.68193	2610020J05Rik	0.000001	-1.573
B020004J07Rik	0.000011	1.68097	Rmnd5b	0.004176	-1.57377
D9Ertd280e	0.000188	1.68095	Krt10	0.000579	-1.57382
LOC100045300	0.001497	1.68031	Hspa9	0.000954	-1.57391
Upk2	0.000040	1.68013	Fxyd6	0.000084	-1.5753
0610011F06Rik	0.000003	1.67939	Rnf17	0.002178	-1.57593
Rras	0.000055	1.67822	AI747699	0.000332	-1.57683
S100a11	0.000197	1.67609	Bahcc1	0.001109	-1.57695
H2-Q8	0.000186	1.67567	Mical1	0.000049	-1.57713
Tmem41a	0.000171	1.67387	Mgea6	0.003903	-1.57723
Tspan7	0.000369	1.67307	Slc6a15	0.000050	-1.57734
EG381936	0.000020	1.6724	Tbc1d15	0.003142	-1.57775
Bik	0.000065	1.6715	Angel2	0.000444	-1.5781
Dusp2	0.000157	1.67122	XEDAR EDA-A2R	0.000764	-1.57815
Ramp3	0.000068	1.67042	LOC381200	0.000082	-1.57943
Ccdc3	0.000000	1.67033	Pnrc2	0.000003	-1.5809
B230369L08Rik	0.000654	1.67016	Rcor2	0.000472	-1.58111
LOC100045981	0.000038	1.66894	Ube1c	0.002015	-1.58181
Tst	0.000129	1.66784	Nfs1	0.000002	-1.58241
Nuak1	0.000065	1.6672	2700023E23Rik	0.000481	-1.58246
C030002B11Rik	0.000002	1.667	Ssr2	0.000948	-1.58319
Gpsn2	0.000274	1.66486	Dus4l	0.000496	-1.58357
Cidea	0.000084	1.66463	Ccdc55	0.000185	-1.58588
Trp53inp2	0.000064	1.66396	scl00238693.1_37	0.001462	-1.58772
Trh	0.000255	1.66326	LOC100048330	0.000002	-1.58898
Plcd1	0.003116	1.66246	Uba2	0.000195	-1.59269
Tcfap2c	0.000017	1.66177	Ccnl1	0.000010	-1.59496
Pcp4l1	0.000001	1.66041	Birc2	0.000167	-1.59501
Vgf	0.000127	1.65928	Zfp260	0.000084	-1.59659
Mmp17	0.000014	1.65812	B930030B22Rik	0.000004	-1.59738
Exoc3l	0.001993	1.65777	Msh6	0.000141	-1.59775
Rab25	0.000048	1.65762	Matr3	0.001185	-1.59954
Nkd2	0.000064	1.65757	D1Pas1	0.000058	-1.59961
Lrp11	0.000115	1.65729	Zfp57	0.005024	-1.60226
Trappc2l	0.000310	1.65596	Usp1	0.001841	-1.60232
Fam115c	0.000385	1.65574	Wdsub1	0.001777	-1.60418

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Cib1	0.001471	1.65526	Yme1l1	0.004154	-1.60437
Eml1	0.000002	1.6552	LOC100046343	0.000208	-1.60461
Ptp4a3	0.001130	1.65436	LOC638892	0.003610	-1.60645
Mmp2	0.000192	1.65432	Isy1	0.000013	-1.60869
9430080K19Rik	0.000058	1.65284	4933434E20Rik	0.000028	-1.60899
2810452K22Rik	0.000014	1.65233	Cugbp1	0.000032	-1.60944
Slc25a20	0.000000	1.65199	Sumo3	0.000128	-1.61067
Wdr92	0.000333	1.65091	Pds5a	0.003006	-1.61071
Gprasp1	0.000095	1.65089	Lrrc28	0.000214	-1.61412
Dkk3	0.000001	1.65054	Siah1a	0.000126	-1.61676
Stambpl1	0.000074	1.64996	Rpusd4	0.000029	-1.61679
Krt19	0.000630	1.64985	Ube2g1	0.003331	-1.61689
1700027N10Rik	0.000002	1.64819	Hnrnpa2b1	0.000001	-1.61766
Slc7a7	0.000051	1.64666	Notch4	0.000013	-1.6178
Gmpr	0.003018	1.64591	Rlf	0.000013	-1.61903
D19Wsu162e	0.000007	1.64429	LOC100044776	0.000005	-1.61995
Esrra	0.000009	1.64331	5730453I16Rik	0.000386	-1.62014
Ctsb	0.000884	1.64292	Trip12	0.002743	-1.62055
Tmbim4	0.000023	1.64224	Immp2l	0.001097	-1.62176
Acd	0.000001	1.64136	Tex14	0.000168	-1.62196
Defb36	0.000034	1.6403	Akr1e1	0.000721	-1.62226
Papss1	0.000667	1.64004	A330080J22Rik	0.000333	-1.6228
1700008I05Rik	0.000144	1.63965	E330016A19Rik	0.000000	-1.62281
Sap130	0.000208	1.63931	Eif4a2	0.000062	-1.62304
Krtdap	0.000310	1.63838	ENSMUSG00000053178	0.000063	-1.62398
Arid3a	0.001441	1.63778	Def6	0.000636	-1.62489
Rprml	0.000081	1.63706	Zfp131	0.002598	-1.62532
Cd276	0.000493	1.63693	2210013O21Rik	0.000678	-1.62552
Id2	0.001095	1.63653	Ints12	0.000771	-1.62771
Tm6sf1	0.000089	1.63625	Zfx	0.000013	-1.62911
2310022B05Rik	0.000022	1.63572	Tom1l1	0.000387	-1.63023
Gm817	0.000443	1.63572	4930504E06Rik	0.000001	-1.63135
Ing2	0.000020	1.63558	4930503L19Rik	0.001628	-1.63202
Lpl	0.000032	1.63527	Sulf1	0.000076	-1.63211
Abhd8	0.000047	1.63363	LOC100041430	0.002309	-1.63349
Smtn	0.000194	1.63298	Dusp27	0.004088	-1.63529
1300011L04Rik	0.001239	1.63125	Gfpt2	0.001068	-1.63684
1810020D17Rik	0.000039	1.63113	Etaa1	0.000611	-1.63723
Dctn6	0.001790	1.63109	Stag2	0.000086	-1.63782
Elof1	0.000173	1.63046	Sfrs5	0.000117	-1.63928
BC080695	0.000116	1.62928	Pa2g4	0.002809	-1.64214
Wfdc10	0.000005	1.62794	Smc5l1	0.000896	-1.64786

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Rnf208	0.000013	1.62729	6030408C04Rik	0.000693	-1.64793
Nomo1	0.003002	1.62653	Ptprv	0.000068	-1.64818
Ethe1	0.001337	1.62589	Setd5	0.000031	-1.6484
Comt	0.000218	1.625	Fam120a	0.001267	-1.64842
Pdzk1	0.000001	1.62465	LOC100040573	0.000175	-1.65038
Junb	0.000115	1.62463	1700123A16Rik	0.000424	-1.65092
Syt5	0.000100	1.62445	LOC100041797	0.000614	-1.65116
Fam129b	0.000249	1.62386	Wapal	0.000787	-1.6518
scl0001647.1_23	0.001280	1.62254	C230091E03Rik	0.002237	-1.65209
Tmem50b	0.000000	1.62211	Zscan10	0.000462	-1.65231
LOC100047214	0.000387	1.62211	Nol8	0.000107	-1.65352
Tmem159	0.000037	1.62207	C430020H24Rik	0.000035	-1.65417
Pygl	0.000176	1.62191	4933412A02Rik	0.001695	-1.65459
Csrnp2	0.000073	1.62179	Ddx46	0.004374	-1.6563
Pdrg1	0.003863	1.62165	2900062L11Rik	0.000016	-1.65653
Hpca	0.005732	1.62136	Fzd5	0.000011	-1.65738
Htr5b	0.000009	1.62113	LOC632684	0.003432	-1.65837
LOC638935	0.000239	1.62089	Tlr2	0.000071	-1.65876
LOC100047937	0.000020	1.62055	Eif1a	0.000747	-1.6599
Hspa2	0.000003	1.62005	BC003940	0.000007	-1.66105
AA467197	0.000015	1.61989	LOC100046049	0.000138	-1.66139
Rpl3l	0.000038	1.61937	2610002J02Rik	0.000111	-1.66198
Gale	0.000550	1.61929	Zfp263	0.002182	-1.66231
Litaf	0.000025	1.61845	Ampd1	0.000020	-1.66335
St8sia5	0.000000	1.61822	Ncor1	0.000134	-1.6642
Sertad3	0.000764	1.61798	Clk1	0.000344	-1.66421
Fam108a	0.000521	1.61791	2610101N10Rik	0.000699	-1.66558
Nphp4	0.000001	1.6172	Topors	0.001042	-1.66648
Gdf1	0.000146	1.61667	2310044G17Rik	0.000007	-1.66665
LOC674135	0.000393	1.61597	Mapk1ip1l	0.000481	-1.66853
Acrbp	0.000204	1.61554	Zfp770	0.000006	-1.66922
Ctsz	0.004566	1.61376	Skiv2l2	0.000505	-1.67054
Pfn2	0.000845	1.61376	LOC100043257	0.000700	-1.6708
Greb1	0.000027	1.61319	Sfrs2ip	0.000081	-1.67095
LOC100044298	0.000022	1.61272	Gm428	0.000120	-1.67238
Fgfbp1	0.002712	1.61217	Rsrc2	0.000513	-1.67293
Sema7a	0.000001	1.61026	Tpi1	0.000001	-1.6736
1500031L02Rik	0.000020	1.60883	Cops2	0.000012	-1.67478
Cyb5b	0.000004	1.6084	Tnpo3	0.002053	-1.67581
Gm1467	0.000102	1.60824	Pdha1	0.000041	-1.67591
AA407659	0.000000	1.60818	Mtbp	0.000017	-1.67854
Cst3	0.001536	1.6075	LOC432730	0.002241	-1.67922

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Mtch1	0.000004	1.60707	Seh1l	0.000000	-1.6801
Cpe	0.000085	1.60665	Trim8	0.000253	-1.68151
Scamp1	0.000004	1.60543	Patz1	0.000035	-1.68166
Abca3	0.000005	1.60518	Aasdh	0.000002	-1.6824
Acpl2	0.000000	1.60509	Stc2	0.000034	-1.68282
Dscr1	0.000008	1.60436	Lars	0.000550	-1.68302
Tm2d2	0.000809	1.60382	Set	0.000219	-1.68321
Abp1	0.000045	1.60315	Morc3	0.004458	-1.68761
E430003J01Rik	0.004452	1.60049	Ptcd3	0.001145	-1.68765
D430019H16Rik	0.000006	1.60003	Cbx3	0.001909	-1.68828
Cib2	0.000094	1.59975	Atad1	0.001565	-1.6898
Mrpl28	0.000993	1.59807	Zfp473	0.000177	-1.69404
Plekhg5	0.000928	1.59718	Rpp38	0.000079	-1.69521
Dpysl5	0.000001	1.59694	Tada2l	0.000003	-1.69679
1810046J19Rik	0.002082	1.59641	Acp6	0.000490	-1.69688
Bola2	0.000767	1.59557	Zmym1	0.000703	-1.69715
Pdlim1	0.000218	1.59555	Fignl1	0.000249	-1.69917
LOC640972	0.000276	1.59231	Taf5l	0.000117	-1.69923
2210408F11Rik	0.000003	1.59181	2610028H07Rik	0.000769	-1.70066
Nrtn	0.000063	1.59144	B930007L02Rik	0.001318	-1.70082
Defb30	0.005360	1.59094	Hspd1	0.000083	-1.70594
Apbb1	0.000228	1.59029	2310045K21Rik	0.004054	-1.70603
2010317E24Rik	0.000119	1.58594	Wdr5	0.000061	-1.70988
Fhl2	0.000335	1.58537	Arf6	0.002130	-1.71067
Tmem14c	0.000015	1.58489	Gtf3c3	0.000014	-1.71119
LOC433943	0.000523	1.58436	Arhgef18	0.000005	-1.71315
Pafah1b3	0.002279	1.58384	Sall2	0.000020	-1.71481
E2f6	0.000011	1.58377	Gtf2h1	0.000350	-1.71552
Slc29a4	0.000005	1.58333	Laptm5	0.000037	-1.71597
1700014N06Rik	0.000008	1.5831	Cd68	0.002574	-1.71787
1810013D10Rik	0.003151	1.58306	B930014J03Rik	0.004319	-1.71805
Igfbp4	0.000002	1.58208	Nvl	0.000074	-1.71911
scl000416.1_19	0.000060	1.5819	2010009J12Rik	0.004047	-1.72136
Fkbp11	0.001154	1.58101	Bckdha	0.000040	-1.72307
Gng13	0.004133	1.58087	Kdelc1	0.000067	-1.72873
Mxra7	0.000061	1.58037	Gm129	0.000118	-1.73167
Emp3	0.000603	1.57934	Apobec1	0.000044	-1.73294
LOC100046883	0.000122	1.5792	Dis3l	0.000513	-1.73381
Creg1	0.000014	1.5782	Phf20	0.000455	-1.73631
2410164B09Rik	0.004411	1.57795	1700019D03Rik	0.000282	-1.74133
Abcc3	0.000069	1.57742	Schip1	0.000213	-1.74156
Pop5	0.000457	1.57542	Rrp1b	0.000001	-1.74158

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Itfg3	0.000396	1.57539	Ccdc77	0.000097	-1.74237
Tnfaip8	0.000948	1.57525	1110034G24Rik	0.000169	-1.74237
BC026585	0.000743	1.5742	Smarcad1	0.001039	-1.74453
Ripk3	0.000352	1.57252	Ifitm3	0.002198	-1.74578
H2-T23	0.000401	1.57233	Utx	0.001329	-1.74814
Lmna	0.000090	1.57124	Tmem79	0.000033	-1.7511
Hmha1	0.001295	1.57109	ENSMUSG00000068790	0.000188	-1.75505
Tmem130	0.000023	1.57036	Etv5	0.000152	-1.76078
Endod1	0.000256	1.57022	Fusip1	0.000201	-1.76247
Dner	0.000015	1.57008	Hnrnpf	0.000112	-1.766
LOC100044204	0.000020	1.57007	Rev1	0.000984	-1.77378
Anxa11	0.000067	1.56839	Usp28	0.001810	-1.77567
Nefm	0.000449	1.56784	Hcfc1	0.000909	-1.77665
Oat	0.000189	1.56742	Nus1	0.000011	-1.77894
Klk1	0.001866	1.56721	Scarb2	0.000001	-1.78172
Stxbp1	0.000019	1.56702	Bcl7a	0.000005	-1.78532
Prkd2	0.000049	1.56699	LOC673578	0.000822	-1.79252
2400006N03Rik	0.003750	1.56603	1700030K09Rik	0.000008	-1.79271
Stard8	0.000001	1.56553	Akap12	0.000622	-1.7936
Esam	0.000468	1.56485	Dnajc7	0.002038	-1.79482
5033414D02Rik	0.000184	1.56462	Ephx2	0.000356	-1.79529
3110001P07Rik	0.003078	1.56436	2410137M14Rik	0.000132	-1.79658
Tnni2	0.000214	1.56332	E130102H24Rik	0.000078	-1.79735
Cdo1	0.002032	1.56295	Fubp1	0.000019	-1.79805
Rnase4	0.000088	1.56111	LOC675933	0.003904	-1.80128
Hn1	0.000067	1.56085	Fus	0.003855	-1.80799
Hspb1	0.000104	1.55795	LOC382010	0.000006	-1.81042
Taf9b	0.000001	1.55788	Nanog	0.000978	-1.81258
Furin	0.000028	1.55744	Eif2s3x	0.000936	-1.81325
Pex16	0.002591	1.55729	Csde1	0.000018	-1.81391
Rab3a	0.000014	1.5561	1500011K16Rik	0.000089	-1.81704
9330175B01Rik	0.000655	1.55543	Nol5a	0.000355	-1.8187
Tmem66	0.000005	1.55519	6330534C20Rik	0.000187	-1.81873
Tmem147	0.000872	1.55491	Sirt1	0.000173	-1.82357
Deb1	0.002349	1.5547	2810403A07Rik	0.000000	-1.82606
Ebpl	0.004288	1.55466	2410081M15Rik	0.000001	-1.82611
Elovl1	0.000104	1.5546	LOC100041567	0.000947	-1.82885
Aplf	0.000667	1.55404	BC019806	0.000104	-1.83012
Plaur	0.000887	1.55355	Tceal7	0.000130	-1.83234
Ctdspl	0.000028	1.55349	EG627299	0.000223	-1.83463
1700020N15Rik	0.000870	1.55335	Pus3	0.000021	-1.84333
Prkaca	0.001975	1.55222	Trib3	0.000006	-1.84943

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Acot1	0.000008	1.55143	Lrig3	0.000011	-1.85168
Pnma2	0.000087	1.55136	LOC100046744	0.000032	-1.862
Pard6g	0.000240	1.55042	Msc	0.000015	-1.86361
Ppif	0.000246	1.55021	8430410A17Rik	0.000055	-1.8651
Atp6v0b	0.000007	1.55	Fancd2	0.000011	-1.86519
Mknk2	0.000188	1.54972	LOC100046401	0.001864	-1.86874
2810011L19Rik	0.000163	1.54935	2610019E17Rik	0.001649	-1.86967
2310047M10Rik	0.000306	1.54931	Pkd2	0.000537	-1.86995
Slc17a7	0.004298	1.5481	Hsd17b1	0.000079	-1.87125
Pts	0.000339	1.54586	Cep55	0.000148	-1.87781
Hip1r	0.000011	1.54568	Gja1	0.000033	-1.87991
Camkv	0.000651	1.54543	Slc2a1	0.000003	-1.87994
Rabl5	0.003411	1.54537	Rn18s	0.000068	-1.88727
LOC100047863	0.000191	1.54493	Ubxn2a	0.003022	-1.88952
D630003M21Rik	0.000007	1.54492	Mettl4	0.000006	-1.89075
Dync2li1	0.001330	1.54332	Lace1	0.000192	-1.89183
Mgst1	0.000157	1.54329	2810026P18Rik	0.000016	-1.89591
Gtf3a	0.000471	1.54258	LOC633016	0.000118	-1.89805
2410076I21Rik	0.000812	1.54198	Hmox1	0.000016	-1.90205
Fbxo21	0.000226	1.54147	BC025546	0.000010	-1.90445
Mrpl43	0.000059	1.54129	AU021838	0.000353	-1.90584
Cnn2	0.000529	1.5411	Epb4.1l4a	0.000015	-1.90685
Smap2	0.000248	1.54044	Smpdl3b	0.000036	-1.90801
Sort1	0.000034	1.54012	Fiz1	0.000013	-1.90928
Pnpla2	0.000483	1.53944	Bcap29	0.000015	-1.91553
Ypel5	0.000022	1.53907	LOC668183	0.000992	-1.91792
Slc4a2	0.000085	1.53907	Mia1	0.000011	-1.91809
2610018I05Rik	0.000043	1.53899	Gbx2	0.000006	-1.92344
Pcbd1	0.001563	1.53834	2600005C20Rik	0.000026	-1.93782
Lrrc15	0.000154	1.53828	Wdr43	0.000015	-1.93955
Popdc3	0.000147	1.53746	Emp1	0.000016	-1.94158
3110079O15Rik	0.000333	1.53691	Frrs1	0.000058	-1.94565
Svop	0.000145	1.53668	Ccno	0.000009	-1.95118
Nnat	0.000001	1.53666	C330036H15Rik	0.000002	-1.95816
Ela1	0.000006	1.53648	Mid1ip1	0.000073	-1.96519
Ephx1	0.000449	1.53631	Rpp25	0.000014	-1.97009
Kcnab2	0.000010	1.53513	Slc28a1	0.000104	-1.97251
Tspan2	0.001028	1.53512	Cwf19l2	0.000005	-1.97561
Triobp	0.000212	1.53471	Caprin1	0.005149	-1.98166
Cldn11	0.000235	1.53462	Zcwpw1	0.000002	-1.98501
Gnal1	0.000124	1.53289	6720463L11Rik	0.000000	-1.98807
Srf	0.000015	1.53255	Thumpd3	0.000023	-1.99004

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Ppl	0.000001	1.5321	Armcx1	0.000502	-1.99141
Neurl	0.000131	1.53135	Setd1b	0.000067	-1.99181
Slc25a1	0.000034	1.53088	Cbr3	0.000025	-2.01115
Rtn2	0.000712	1.52901	5730528L13Rik	0.000060	-2.01163
Kif1a	0.002400	1.5282	scl0001487.1_50	0.000013	-2.01727
Susd4	0.000432	1.52763	LOC100045617	0.000005	-2.01759
2310007A19Rik	0.000101	1.52719	Myo1f	0.000001	-2.03682
Llgl2	0.000040	1.52703	Cth	0.000267	-2.0385
Ostm1	0.000271	1.52699	Enox1	0.000044	-2.04007
LOC673556	0.000082	1.52659	Zfp428	0.000014	-2.04469
Jak1	0.000046	1.52653	Prpf40a	0.000078	-2.04639
Uap1l1	0.005916	1.52634	Thtpa	0.000011	-2.05816
2300002D11Rik	0.000062	1.52555	Tnfsf12-tnfsf13	0.000003	-2.05981
Cyp4f14	0.000618	1.52538	Slc25a36	0.000038	-2.06456
Ilk	0.000165	1.52518	Tdh	0.000277	-2.07019
Map2k6	0.000000	1.52506	Mid1	0.000040	-2.07626
Galnt10	0.000040	1.52503	Nanogpd	0.000014	-2.07839
Gltp	0.000301	1.52428	Fgf4	0.000052	-2.09785
Alox5ap	0.000350	1.52427	Upp1	0.000004	-2.10195
1110012O05Rik	0.000292	1.52389	5730406M06Rik	0.000017	-2.10743
Dmrtc1c	0.000292	1.52388	5033413D16Rik	0.000001	-2.13437
LOC100048169	0.001699	1.52323	Ktelc1	0.000007	-2.14204
3110001A13Rik	0.000599	1.52291	Gdf15	0.000008	-2.16128
Car12	0.000023	1.52175	Slc7a3	0.000018	-2.1645
Hdc	0.000012	1.52142	Rdm1	0.000056	-2.17217
Plcd3	0.001280	1.52037	Snora65	0.000002	-2.18093
Aacs	0.000031	1.51878	LOC383491	0.000003	-2.23164
App	0.000066	1.51864	LOC100046320	0.003795	-2.24417
Wnt4	0.000144	1.5182	Phc1	0.000007	-2.25489
Insl6	0.002616	1.51819	BC028528	0.000006	-2.27063
Gstp2	0.000054	1.51796	Cxxc6	0.000034	-2.27118
Maged1	0.000222	1.5179	Vegfc	0.000002	-2.28763
Stab1	0.000138	1.51772	Ccnd2	0.000004	-2.29722
Htatip2	0.002768	1.51688	Tera-pending	0.000487	-2.32922
9530064J02	0.000033	1.51675	Zfhx2	0.000008	-2.32941
H19	0.000001	1.51631	Manba	0.000002	-2.33313
Lad1	0.001016	1.51597	BC032203	0.000000	-2.37505
Amigo2	0.000000	1.51519	D130003B22Rik	0.000000	-2.4262
Prkcd	0.000858	1.51481	Gm1967	0.000000	-2.4808
A530057A03Rik	0.000004	1.51336	Epb4.9	0.000000	-2.48301
Mir16	0.000005	1.51322	Spp1	0.000166	-2.50207
Pex6	0.000116	1.51258	Fgf17	0.000001	-2.52125

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Hs3st3b1	0.000004	1.51232	Meis2	0.000003	-2.60559
Tusc4	0.005700	1.51148	Gli2	0.000000	-2.61574
Arhgdib	0.000951	1.51147	Gdf3	0.000001	-2.70091
D15Wsu169e	0.003996	1.51116	E130014J05Rik	0.000110	-2.73198
Fam125a	0.000164	1.51085	Senp3	0.000003	-2.76786
Ly6e	0.000197	1.51075	E430003D02Rik	0.003067	-2.96588
Nid2	0.002352	1.51073	Chac1	0.000000	-3.3549
Lcmt1	0.000024	1.51022	Nupr1	0.000136	-3.76715
Galk1	0.000008	1.50946	LOC208080	0.000000	-4.54358
Plcg2	0.000295	1.50916	LOC100046802	0.000000	-5.41523
Rilpl1	0.002061	1.50797			
Ssbp3	0.000256	1.50666			
Aqp11	0.001615	1.5063			
Crtac1	0.005857	1.50618			
Mgat4b	0.000066	1.50612			
Obox6	0.000040	1.50549			
Espn	0.000022	1.5051			
H1fx	0.000558	1.50508			
EG433923	0.002318	1.50392			
1700123J19Rik	0.001457	1.5033			
Ube2n	0.000005	1.50284			
Sepx1	0.000022	1.50236			
Ap1b1	0.000034	1.50231			
Slc4a1	0.000011	1.50211			
Sep	0.000003	1.50171			
Sdf2l1	0.005391	1.50082			
Tuba4a	0.000242	1.49902			
Ng23	0.002283	1.49897			
Dusp1	0.001271	1.49861			
Slc13a4	0.003217	1.49695			
Igf2r	0.000299	1.4968			
Hk1	0.000971	1.49593			
Itpr3	0.001146	1.4945			
D0H4S114	0.000411	1.49427			
Cyb5r3	0.000441	1.49423			
Fos	0.000978	1.49421			
Zfyve21	0.001579	1.49346			
LOC380878	0.000154	1.49297			
Zcchc18	0.000035	1.49285			
Gstm1	0.000029	1.49283			
Unc119b	0.000058	1.49162			
Mns1	0.000142	1.49087			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Edem2	0.000181	1.49			
Tph2	0.000028	1.49			
Tpcn1	0.000095	1.48965			
Mfsd7c	0.000041	1.48901			
Bloc1s1	0.003141	1.48727			
Prkcz	0.000025	1.48679			
Ptrf	0.000099	1.48634			
9630007E23Rik	0.000083	1.48591			
Pde2a	0.000337	1.48579			
Rhog	0.000357	1.48571			
Jtv1	0.001579	1.48553			
Scly	0.000209	1.48466			
Hes6	0.000280	1.48451			
Mvd	0.004084	1.4842			
Adamts7	0.000244	1.48417			
Lasp1	0.000532	1.48364			
Rpl22	0.000030	1.48263			
6230427J02Rik	0.000203	1.48261			
Lzts2	0.000267	1.48181			
Gmds	0.000131	1.48161			
Acss2	0.000028	1.48134			
Wnt7b	0.000058	1.48063			
Zbtb46	0.001420	1.4804			
1110046J11Rik	0.000010	1.4803			
Ell3	0.000080	1.48024			
Brms1	0.000092	1.47952			
Ift20	0.000380	1.47889			
Pgc	0.001391	1.4783			
2210011C24Rik	0.000750	1.47816			
Zbtb32	0.000596	1.47785			
Coro1c	0.000595	1.47765			
Flot1	0.000146	1.47729			
Ybx1	0.000892	1.47697			
LOC100045343	0.000039	1.47671			
LOC332788	0.000733	1.47568			
1700108L22Rik	0.001943	1.47532			
Yaf2	0.001150	1.47486			
Col7a1	0.000021	1.47372			
Ptges	0.000456	1.47364			
Gstm5	0.000865	1.47356			
1200003C05Rik	0.000900	1.47351			
Lrpap1	0.000031	1.47302			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Chi3l1	0.000488	1.47273			
Dpp3	0.000005	1.47197			
Tcea3	0.000518	1.47152			
Lrrc8a	0.003280	1.47137			
LOC624662	0.000576	1.47127			
Gm1006	0.000028	1.47122			
C330002I19Rik	0.000019	1.4711			
Acadvl	0.000036	1.4709			
Sult4a1	0.000047	1.46991			
Pdgfb	0.000113	1.46978			
Tesk1	0.000021	1.46969			
Gps2	0.000002	1.46836			
Rabl2a	0.000453	1.46745			
Tspan33	0.004187	1.46664			
2210410E06Rik	0.000298	1.46628			
4933439C20Rik	0.000077	1.46582			
Gcap27	0.000558	1.46553			
Rab3ip	0.004728	1.46535			
OTTMUSG00000010552	0.000602	1.46463			
Glrx	0.000648	1.46462			
Nudt18	0.000250	1.46457			
Acbd4	0.000223	1.46379			
Pyy	0.001621	1.46333			
Tm4sf5	0.000695	1.46329			
Kctd17	0.000191	1.4631			
Stxbp2	0.000259	1.46227			
Mtif3	0.003195	1.46184			
1700086L19Rik	0.000200	1.46142			
Tpm4	0.001216	1.46106			
4930511J11Rik	0.000438	1.46027			
Efemp2	0.003600	1.45996			
1500009L16Rik	0.004152	1.45995			
BC039093	0.003017	1.45987			
Scara5	0.000127	1.45975			
Zdhhc12	0.000568	1.45796			
Wbp2nl	0.000944	1.45783			
Limch1	0.005879	1.45772			
Tmed10	0.000010	1.45758			
Ctnnal1	0.000872	1.45583			
Rnaset2	0.005177	1.45572			
Vps53	0.001241	1.45444			
Prr5	0.000866	1.45327			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Atp1b2	0.002372	1.4531			
Suclg1	0.000281	1.45254			
Brp17	0.001444	1.45211			
Sar1a	0.004754	1.45194			
Fhl1	0.000202	1.45183			
Gaa	0.000125	1.45153			
Tro	0.000104	1.45147			
Optn	0.000054	1.45121			
Enpp5	0.000306	1.45093			
Xpr1	0.000020	1.45075			
Bcas1	0.001045	1.45065			
Tnfrsf12a	0.001553	1.45062			
ENSMUSG00000054212	0.000049	1.45058			
D030035F05Rik	0.000483	1.45058			
Tbcb	0.000029	1.45045			
Tspan14	0.000105	1.4504			
Srprb	0.004595	1.45021			
Idb2	0.002276	1.44963			
Unc119	0.000067	1.44867			
Atp6v1c2	0.000123	1.44865			
Itm2b	0.000017	1.44861			
Tmem184b	0.000002	1.44846			
Ccbl1	0.000307	1.44846			
Txndc5	0.000315	1.44825			
Bcl9l	0.000951	1.44814			
Tmem141	0.001340	1.44802			
Tmem86a	0.000920	1.44798			
Moxd1	0.000018	1.44768			
1700006H02Rik	0.000068	1.4473			
2310005E10Rik	0.001738	1.44541			
Sh3pxd2b	0.000067	1.44464			
Zcchc12	0.000034	1.44404			
Bad	0.000271	1.44388			
Plac8	0.000931	1.44378			
4930431B09Rik	0.000014	1.44297			
Myo1e	0.001239	1.44294			
Bahd1	0.000057	1.44253			
5031425E22Rik	0.000231	1.44212			
Gde1	0.000179	1.44192			
Mad2l1bp	0.000029	1.4416			
Cmas	0.001045	1.44148			
LOC639931	0.002239	1.44068			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
1500001M20Rik	0.000052	1.44041			
Pygb	0.000346	1.4399			
Reg1	0.000521	1.43985			
D13Ertd608e	0.000457	1.43975			
Pigx	0.002354	1.43943			
Cklf	0.000000	1.43917			
Pepd	0.000277	1.43812			
2310047A01Rik	0.000130	1.43808			
Lpcat3	0.000744	1.43765			
LOC633360	0.000052	1.43699			
Spsb4	0.000022	1.43694			
Ankrd37	0.000050	1.43677			
Mrps26	0.000198	1.43673			
Lypd3	0.000794	1.43651			
Dpysl2	0.000123	1.43644			
LOC381860	0.000273	1.43537			
Usp2	0.000023	1.43502			
Tex261	0.000443	1.43496			
Arpc4	0.000733	1.43483			
Wnt7a	0.000004	1.43474			
Pes1	0.001235	1.43447			
Aldh1l1	0.001531	1.43439			
Gpsm1	0.000057	1.43433			
Rrbp1	0.001172	1.4336			
Fgfr2	0.000002	1.43263			
Psme1	0.000611	1.43237			
Cdk5r1	0.000002	1.4319			
Apoa1bp	0.003734	1.43144			
scl0002540.1_6	0.002623	1.43107			
Sirt2	0.004023	1.43107			
Qpct	0.000328	1.43101			
LOC329984	0.000049	1.43063			
Rfc3	0.000583	1.43011			
Fmnl3	0.000033	1.43002			
Prkacb	0.005806	1.42905			
Arf2	0.000249	1.42893			
Trim41	0.000824	1.42886			
Hdac5	0.000021	1.42883			
Snai3	0.002149	1.42876			
LOC100046918	0.002278	1.42734			
Ccnjl	0.000372	1.42712			
4732471D19Rik	0.002265	1.42708			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Stard5	0.000083	1.42662			
Defb42	0.001868	1.42654			
A830080H07Rik	0.000037	1.42644			
Stac2	0.000143	1.42622			
Ngfrap1	0.002688	1.42598			
Asb2	0.000086	1.42592			
Acsl6	0.000010	1.4249			
0610037M15Rik	0.001313	1.42429			
Rhof	0.000001	1.42414			
Atp6v1e1	0.001110	1.42407			
Tomm34	0.000157	1.42354			
Lman2	0.003770	1.42323			
3010026O09Rik	0.001143	1.42267			
Taldo1	0.005108	1.42254			
Diras2	0.000717	1.42192			
Col18a1	0.000455	1.42186			
Cldn4	0.000642	1.42148			
Ctsl	0.000359	1.42124			
Pscd3	0.001861	1.42119			
Tuba3a	0.001519	1.42086			
Bcl2l11	0.000229	1.41999			
Cgrrf1	0.000675	1.41929			
2310014G06Rik	0.000036	1.41898			
Sord	0.001990	1.41849			
Scand1	0.003427	1.4179			
Nr6a1	0.000127	1.41783			
Fxn	0.000303	1.41732			
Crygs	0.000482	1.41672			
Fam110c	0.002471	1.41658			
LOC384348	0.002849	1.41647			
Socs3	0.000822	1.41544			
Tex19.1	0.002570	1.41534			
Ndufa10	0.002545	1.41516			
Ndufa12l	0.002513	1.41505			
Mrpl4	0.002374	1.41448			
Dap	0.001617	1.41424			
Dnajb11	0.002998	1.41334			
Ndufa12	0.000357	1.41319			
Fam148c	0.000541	1.41318			
9430038I01Rik	0.000303	1.413			
Arc	0.000753	1.4129			
D15Mit260	0.000000	1.41257			

Day0 vs. Day3					
Gene Symbol	**p-value**	**FC Up-regulated**	**Gene Symbol**	**p-value**	**FC Down-regulated**
Ttc39a	0.000019	1.41249			
F2rl1	0.000067	1.41219			
Sep-15	0.000947	1.41207			
Ccdc92	0.000048	1.41199			
Ptpn18	0.004348	1.41183			
2310061C15Rik	0.001954	1.41121			
Dapk2	0.000006	1.41071			
Hmgcl	0.000003	1.41071			
Pemt	0.000115	1.41051			
Agap1	0.000215	1.40999			
Atp6v0d1	0.000138	1.40992			
Rab15	0.002679	1.40935			
A430088H15Rik	0.000170	1.40858			
Gm347	0.002074	1.40854			
Chga	0.000452	1.40825			
En1	0.000176	1.40816			
Slc1a1	0.000239	1.40807			
H2-Ke6	0.001147	1.40804			
Rnasek	0.001188	1.40795			
Adrb2	0.000928	1.40795			
Gm2a	0.002032	1.40793			
Lgals6	0.002790	1.40778			
Fxyd5	0.002805	1.4071			
Prnp	0.000364	1.40693			
2610014I16Rik	0.000417	1.4063			
Rbm38	0.000120	1.4058			
Areg	0.000083	1.40513			
B020031M17Rik	0.005717	1.40475			
Impa2	0.000131	1.40468			
Reep3	0.000095	1.4038			
2010007H12Rik	0.000012	1.40309			
6230400G14Rik	0.000097	1.40222			
A530050D06Rik	0.005792	1.40185			
LOC236277	0.000074	1.40176			
Aard	0.000147	1.4015			
Plvap	0.001957	1.401			
Srxn1	0.000092	1.40025			
1700052O22Rik	0.003143	1.4			

Table 6:List of genes deregulated ≥1.4-fold (adjusted P < 0.05) in C vs. C+RA, and KO vs. KO+RA.

C vs. C+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Cyp26a1	13.06429623	Otx2	2.697001444
Aurkc	4.518015463	Chst1	2.106977307
Cdx1	3.700597632	Ndrl	2.032011113
Rarb	2.607989595	Ndrg1	1.935035031
Camk2n1	2.718890815	Slc40a1	1.883861451
Hoxb1	2.235125425	Socs2	1.866457438
Zfhx2	2.090949689	Enc1	1.738388809
Rhobtb1	1.88205112	Zfp459	1.734138458
Ppbp	2.479178951	Gjb3	1.729839525
Ppl	1.913943321	Fst	1.716368959
Folr1	1.709015971	Aire	1.683127342
Raet1b	1.754149208	Igfbp5	1.676517832
Rin2	1.747737325	Fgf17	1.666300735
Gabarapl2	1.696408063	Ly6g6e	1.622791517
Cotl1	1.624153367	Kndc1	1.619921682
Mdk	1.638335728	LOC100044968	1.617399166
Cpm	1.558971644	Ddx58	1.587240885
Irak2	1.639268018	Zfp42	1.561294953
Aebp2	1.607079892	1190003J15Rik	1.55774474
Ndp52	1.514332028	Slc6a15	1.55406297
Raet1c	1.504392531	Rhbdf1	1.54955408
Gpx4	1.497309501	Msrb2	1.535414041
Tcfap2c	1.537221096	Gcnt2	1.535312216
Sesn1	1.517466944	Fgf5	1.532526037
Cd97	1.498892399	Plec1	1.529718239
Clgn	1.500938203	Cdc42ep4	1.519443827
Erf	1.454985254	Armcx1	1.503802654
Pml	1.442750871	Dnajc6	1.501432877
Pvrl2	1.410815609	Pcyt1b	1.489970394
Pgpep1	1.406655363	Cdc42ep5	1.484934868
Atp2a2	1.570621279	Spata13	1.482432476
Csnk	5.96921548	Cav1	1.481954461
Csn3	5.804414844	Klf6	1.480816898
Hoxa1	4.801685941	Zfp710	1.472437896
Aqp3	4.718720212	Slc30a3	1.4718655
Stra8	4.675900346	2810022L02Rik	1.464321476
Tal2	3.132498647	Ghr	1.461825044
Tle6	2.629599959	2310043N10Rik	1.459910075
Hoxa5	2.389604451	Klf4	1.458504806
Hoxb4	2.33330503	Igsf9	1.458187529
Meis2	2.323009518	Egr1	1.456721746
Wfikkn1	2.320491498	Dapk1	1.448027082
Rbp1	2.26975113	Cxcl1	1.445129565
Gpr124	2.233321213	Fzd5	1.444392783
Mrg1	2.155800597	En2	1.444366726
Rgma	2.138962431	Sgk1	1.444297678
Dppa2	2.128485266	En1	1.441876704
Grasp	2.098080663	Fam129b	1.441490829
Gata6	2.085329687	Klf9	1.427881647

C vs. C+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Hoxb2	2.084743007	1700019H03Rik	1.421908207
Notch4	2.037366622	Socs3	1.421889332
Dlx3	2.01956158	1110012J17Rik	1.421869718
4933421H10Rik	2.016756288	Rbm35a	1.419212469
Hoxb5	2.006265957	Dapp1	1.418662029
Tgm2	2.003796683	1700019D03Rik	1.417527411
Lefty1	1.962439333	Tle4	1.416252243
8030467N07Rik	1.951956814	Ahnak2	1.415737487
Nrip1	1.939515007	Nefm	1.412637043
Rhbdl3	1.936319088	Id4	1.409091626
Foxn4	1.924186008	Cd59b	1.407515167
Dleu7	1.911251335	Slc29a1	1.406235915
Zmym3	1.904159538	Nid2	1.402000062
Meis1	1.888858069	Pde1b	1.84809482
Irf5	1.88811861	Slc27a2	1.702333223
Foxp1	1.877774802	Dnmt3l	1.749279434
Ltbp3	1.874900175	Ildr1	1.594741839
Rage	1.872276392	Zic3	1.573843764
Dusp9	1.871000377	Lrrc34	1.551483342
H2-Bl	1.860964989	F2rl1	1.456398322
D16Bwg1494e	1.85423442	Dusp4	1.450917521
Pdyn	1.839523864	Zfp36l1	1.451750561
5930418K15Rik	1.782647146		
Pptc7	1.780752617		
Wnt3a	1.763818414		
2600009P04Rik	1.75912626		
LOC100047268	1.75304771		
Porcn	1.742484952		
Myo1f	1.739638233		
LOC100040525	1.739442776		
Cd37	1.739346532		
Plk3	1.738545197		
G630016D24Rik	1.723462648		
Ccdc88b	1.721212107		
Spsb1	1.715368044		
Nt5e	1.689708173		
Nphp4	1.688340473		
Gm22	1.679959453		
Ier5l	1.677408419		
Adfp	1.675010883		
Mmd	1.658736805		
Pmp22	1.632536711		
Ppap2b	1.618969661		
Prrt3	1.617768837		
Fcgrt	1.615399714		
Enpp4	1.611793956		
Eras	1.609246251		
D230007K08Rik	1.601750078		
Nudt4	1.598304902		
Ap3b2	1.593214389		
Tinagl	1.584099959		
Llgl2	1.581713047		
Atp11b	1.579304012		
D17H6S56E-5	1.569820304		

C vs. C+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Raet1a	1.568832932		
Cidea	1.56620483		
Nr6a1	1.561588671		
LOC100047651	1.557103966		
BC019806	1.556888222		
Epb4.1l1	1.547937609		
6330442E10Rik	1.544818818		
2700038C09Rik	1.53564068		
scl0004020.1_31	1.529109535		
Pacsin1	1.527528263		
Uck1	1.52690082		
D14Ertd668e	1.526359066		
LOC676420	1.524990101		
2200001I15Rik	1.524810739		
Twf2	1.52139231		
Tnfrsf13c	1.521274632		
Tinagl1	1.513790443		
Kit	1.513446221		
Ski	1.50453728		
B3gnt7	1.499093877		
LOC435145	1.498303545		
Cxcl10	1.493122529		
C2cd2l	1.491332479		
Ccdc120	1.487121885		
Gca	1.480531759		
Dok4	1.480038722		
Tmtc1	1.47850134		
Elavl3	1.474573912		
3110001A13Rik	1.473902536		
Slc38a8	1.466882383		
Elovl6	1.466657131		
Tmem166	1.463181029		
Tmem181	1.460971456		
Gpr114	1.460726134		
Dock6	1.460453022		
Smyd2	1.460288315		
Aifm2	1.459094161		
Myl7	1.454004269		
Dusp14	1.453741426		
Nxn	1.45228287		
LOC100046741	1.451861861		
Atf3	1.450271761		
Synpo	1.446406002		
Tmem132e	1.445192741		
Dpp4	1.443850795		
6330534C20Rik	1.43727911		
Zfp809	1.43547117		
Sema4g	1.43539525		
Gdf15	1.433494132		
Zfhx3	1.430012938		
4631416L12Rik	1.428414466		
Fbp2	1.427371329		
Lnx2	1.42612017		
Efnb1	1.422343556		

C vs. C+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Oaz2	1.420945128		
5730593F17Rik	1.413851779		
Ttc7b	1.410626127		
Wdr6	1.410519707		
1700028I16Rik	1.409581063		
Mtf2	1.40485765		
Dgkz	1.404832776		
Myo10	1.404542623		
Fbxo27	1.403628667		
Snai1	1.401191235		

KO vs. KO+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Cyp26a1	9.788999727	Pdk4	1.460454128
Csnk	9.53296688	Ndrl	1.835072063
Csn3	9.245236816	Ndrg1	1.837279788
Hoxa1	1.51763988	Sp5	1.944351241
Aqp3	4.242441074	Socs2	1.636267034
Stra8	3.10576649	Slc27a2	1.53616021
Aurkc	1.540476807	Igfbp5	1.846371394
Cdx1	2.69236107	Dnmt3l	1.555414976
Rarb	1.700682662	Enc1	1.584276326
Ppbp	6.758186399	Zfp459	1.74859238
Camk2n1	1.679619364	Gjb3	1.703621665
Tle6	2.879699256	Fst	1.743448594
Hoxb4	2.376718042	Aire	1.447406267
Wfikkn1	2.229497114	Zic3	1.494688251
Gpr124	2.241493948	LOC100044968	1.596780644
Rgma	1.492665836	Lor	1.486358822
Rhobtb1	1.444719638	Zfp42	1.891881106
Dlx3	2.764453854	Rhbdf1	1.448229962
4933421H10Rik	1.563857608	Gcnt2	1.643488548
Hoxb5	1.476767509	F2rl1	1.678994973
Tgm2	2.188088697	Ildr1	1.437455073
Ppl	2.268464763	Cav1	1.408032884
8030467N07Rik	2.12283515	Klf6	1.566424879
Nrip1	1.914527871	Dusp4	1.600960027
Rhbdl3	1.600979376	Zfp710	1.633896921
Zmym3	1.485906202	Zfp36l1	1.401884558
Ltbp3	2.163537555	Igsf9	1.431930694
Dusp9	2.151725068	Egr1	1.648956294
H2-Bl	1.918329982	Sgk1	1.563513896
5930418K15Rik	1.542448612	Fam129b	1.455163515
Pptc7	1.456924757	Klf4	1.469625686
Porcn	1.534129522	Socs3	1.980302793
LOC100040525	1.404298116	Slc29a1	1.44977191
Cd37	1.603961343	Ier3	1.483518302
Ccdc88b	2.117239308	C130035G06Rik	1.487919878
Mdk	1.536982108	Bmp4	2.139120308
Nphp4	1.597369012	Cited2	1.589316832
Cpm	1.730314978	Thy1	1.461895433
Gm22	1.894365215	Enox1	1.433456716

KO vs. KO+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Mmd	1.660800383	Vgf	1.877759027
Atp2a2	1.4691846	A730027B03Rik	1.539546604
Pmp22	1.941294443	Klf5	1.657548073
D230007K08Rik	1.502752016	Nrp2	1.542082071
Tinagl	1.40560688	Slc7a3	1.404047192
Llgl2	1.433627109	Fgfbp1	1.563375914
D17H6S56E-5	1.714225138	LOC100048710	1.577226632
Cidea	1.411723903	Stx11	1.443531871
Aebp2	1.444275377	Hs3st3b1	1.41078325
scl0004020.1_31	1.483902	Nppb	1.809377761
LOC676420	1.650028392	Pmaip1	1.526421492
Tinagl1	1.671577459	Zfp57	1.598617507
Kit	1.783697972	Bdnf	1.470932348
B3gnt7	1.592245369	Tgif1	1.612376202
Letmd1	1.682982647	Zscan10	1.499483952
Erf	1.493841279	Zfp296	1.465045724
Elovl6	1.406485979	Tcstv3	1.442509045
Dock6	1.457091461	Tpbg	1.475425659
Atf3	1.742878703	Trim25	1.50927177
Synpo	1.775201771	Calca	1.516987436
Sema4g	1.712656252	Rem2	1.53144964
Zfhx3	1.435059057	Lmna	1.422817521
Ttc7b	1.483556432	Plaur	1.543050431
Mtf2	1.462668458	Esrrb	1.5039306
Pcdh1	1.907195406	LOC100046232	1.453869733
H2-T23	1.523695038	3110040M04Rik	1.460254398
Cdv3	1.770856208	Krt14	2.570029373
Plcd3	1.506712907	Ankrd1	1.415011264
Plvap	1.582045436	Arc	1.428035081
Skil	1.614509285	Timp1	1.421489806
BC020108	1.942767617	Col6a3	1.423582787
Stra6	1.76690039	Tgfb1i1	1.591220372
Csnk1e	1.457735432	Cd274	1.552539745
Smox	1.404923994	Crct1	1.999173059
EG630499	1.402617208	Nts	1.877064161
Plekhg6	1.536955122	Sfn	1.552988906
Mkrn1	1.595861608	Lce1f	1.680450805
Gse1	1.61920676	Gata3	1.505926355
Mela	1.556085043	Ctgf	1.445190564
2310016C08Rik	2.072242447	Cldn11	1.898177409
Ngfr	2.095015956	D13Ertd608e	1.457800276
Pdgfrb	1.527973697		
LOC666652	1.587848459		
Scd1	1.856538037		
Fzd7	1.417187486		
Ptpn21	1.498701706		
Arg1	1.655837515		
Smad3	1.580998482		
Dgkz	1.400078455		
Centd3	1.840047942		
2510009E07Rik	1.442602436		
Arrdc2	1.933980367		
Cebpb	1.402763725		
Ppp2r1b	1.503407341		

KO vs. KO+RA			
Gene Symbol	**FC up-regulated**	**Gene Symbol**	**FC down-regulated**
Lrig1	1.405743037		
D14Ertd668e	1.410795785		
Cgnl1	1.481332649		
Gbx2	1.947557141		
Chac1	1.516951237		
2310007B03Rik	1.40459634		
Prickle1	1.542986298		
Epn2	1.415401293		
LOC100043671	1.550444976		
Fkbp14	1.659616816		
Baiap2l1	1.876958052		
LOC100044702	1.452478307		
EG546036	1.413575292		
Crybg3	1.583706676		
Pfkfb4	1.749304862		
Ets2	1.442854499		
1700031F05Rik	1.692589193		
LOC100047659	1.524915994		
Slc13a4	1.520863952		
Celsr3	1.57972284		
Rec8	1.468039621		
E130014J05Rik	1.657780773		

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