

Forschungszentrum Karlsruhe

In der Helmholtz-Gemeinschaft

Wissenschaftliche Berichte

FZKA 6806

**Valproic Acid and Peroxisome Proliferator-activated Receptor δ
Are Selective Modulators of a Histone Deacetylase with
Implication for the Transformed Phenotype of
Colorectal Cancer Cells ***

Ping Zhu

Institut für Toxikologie und Genetik

* Von der Fakultät für Chemie und Biowissenschaften der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe

2002

Impressum der Print-Ausgabe:

**Als Manuskript gedruckt
Für diesen Bericht behalten wir uns alle Rechte vor**

**Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe**

**Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)**

ISSN 0947-8620

**Valproic Acid and Peroxisome Proliferator-activated Receptor δ
Are Selective Modulators of a Histone Deacetylase with
Implication for the Transformed Phenotype of
Colorectal Cancer Cells**

Zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
an der Fakultät für Chemie und Biowissenschaften
der Universität Karlsruhe (TH)
genehmigte

DISSERTATION

von

Ping ZHU

aus Shanghai, China

2002

Dekan: Prof. Dr. M. Metzler

- 1. Gutachter:** PD Dr. Martin Göttlicher
- 2. Gutachter:** Prof. Dr. Peter Herrlich

Tag der mündlichen Prüfung: (Tag der Schlußsitzung) 23.10.2002

Abstract

Valproic acid (VPA) has multiple activities, e.g. it is an anti-epileptic drug, it is employed to treat bipolar disorders, it induces birth defects and, in rare cases, it is toxic to the liver. The different activities are likely to follow different mechanisms of actions. Previous findings indicated that VPA and similar compounds that also induce birth defects induce differentiation of F9 teratocarcinoma cells. This differentiation-inducing activity is always associated with activation of Peroxisome Proliferator-activated Receptor δ - (PPAR δ)-dependent transcription. PPAR δ is a nuclear receptor proposed to play an important role in colorectal tumorigenesis. The open questions to be addressed in this thesis were by which mechanism VPA activates PPAR δ -dependent transcription, whether this mode of VPA action has implications for the development of colonic cancer, and what could be downstream targets regulated by PPAR δ .

The first major finding of this study was that VPA is a subset-selective inhibitor of histone deacetylases (HDACs) and, in addition, selectively reduces HDAC2 protein levels. This finding was supported by observations from *in vitro* HDAC assays and from investigations on HDAC expression upon VPA-treatment. At those VPA concentrations that are employed also for therapy of epilepsy VPA preferentially inhibits class I HDACs rather than class II enzymes. In addition to the inhibition of enzymatic activity VPA selectively down-regulates HDAC2 protein levels by inducing ubiquitination and subsequent proteasomal degradation. Since recruitment of corepressor-associated HDAC activities by unliganded PPAR δ is thought to be required for repression of PPAR δ -dependent gene expression, HDAC inhibition is the most likely mechanism by which VPA activates PPAR δ -dependent transcription. More importantly, this finding suggests a potential use of VPA in cancer therapy since inhibition of HDACs is considered to be of therapeutic value in many forms of cancer.

The second major finding was that HDAC2 activity is essential for the transformed phenotype of colorectal cancer cells. This conclusion was mainly

supported by evidence from a colorectal cancer cell line that contains mutant form of the tumor suppressor gene *adenomatous polyposis coli (APC)* and undergoes apoptosis upon restoration of wild-type APC expression. In this cell line HDAC2 expression was found to be repressed by the presence of wild-type APC. Loss-of-function and gain-of-function analyses indicated that HDAC2 is required and sufficient for preventing apoptosis and maintaining the transformed phenotype of colorectal cancer cells. Since elevated levels of HDAC2 were detected in many samples of colorectal carcinomas, HDAC2 is likely also to play a role during pathogenesis of colorectal cancer *in vivo*.

Finally, evidence was obtained that PPAR δ regulates HDAC2 expression either directly or indirectly in murine ES and F9 teratocarcinoma cells. Since PPAR δ is negatively regulated by APC, this transcription factor may be one of the links that mediates overexpression of HDAC2 upon loss of wild-type APC.

In summary, this study revealed specific VPA-dependent repression of selected HDAC activities, both, by inhibition of class I HDAC enzyme activities and targeted degradation of HDAC2 protein. High levels of HDAC2 expression were found to be essential for the transformed phenotype of colorectal cancer cells. Since HDAC2 expression itself might be induced by PPAR δ , and thus by VPA, this drug can be expected to have several and partly opposite effects on HDAC2 abundance and activity in colonic cancer cells. As assessed by cell survival and abundance of HDAC2 levels upon VPA-treatment, induced degradation and inhibition of HDAC activity appear to prevail over a putative induction of HDAC2 gene expression. Therefore VPA appears to be suitable as a therapeutic agent in the therapy of colon cancer patients.

Valproinsäure und Peroxisomenproliferator-aktivierter Rezeptor δ sind selektive Modulatoren einer Histondeacetylase mit Auswirkungen auf den transformierten Phänotyp colorektaler Krebszellen

Zusammenfassung

Das Antiepileptikum Valproinsäure (VPA) und seine teratogenen Derivate induzieren einen spezifischen Typ der Differenzierung von F9-Teratocarcinomzellen und aktivieren selektiv den Peroxisomenproliferator-aktivierten Rezeptor delta (PPAR δ). Bei PPAR δ , dessen Expression durch die APC/ β -Catenin-Signalkaskade reguliert wird, handelt es sich um einen nukleären Rezeptor, von dem man annimmt, dass er eine wichtige Rolle in der Entstehung colorektaler Tumore spielt. Der Mechanismus, über den PPAR δ durch VPA aktiviert wird, ist ebenso unbekannt wie die nachgeschalteten Ziele von PPAR δ im Verlauf der colorektalen Karzinogenese.

Bei der Aufklärung des Mechanismus, welcher der VPA-induzierten PPAR δ -Aktivierung zugrunde liegt, konnte hier gezeigt werden, dass VPA in vitro HDAC-Aktivität hemmt. Im Gegensatz zu Trichostatin A (TSA) jedoch, einem weiteren HDAC-Inhibitor, welcher Histondeacetylasen der Klassen I und II im gleichen Ausmaß hemmt, inhibiert VPA präferenziell Klasse I HDACs, während Klasse II-Enzyme bei den therapeutisch erreichten Konzentrationen kaum beeinflusst werden. Zusätzlich zu dieser Selektivität der direkten HDAC2-Inhibition verringert VPA im Gegensatz zu TSA auch noch die Menge an HDAC2-Protein in den Zellen, jedoch nicht die der anderen Klasse I-Enzyme wie HDAC1 und HDAC3. Dieser Effekt läßt sich auf einen durch VPA-Behandlung selektiv verstärkten Abbau des HDAC2-Proteins über das Ubiquitin-Proteasom-System und nicht auf transkriptionelle oder translationelle Regulation zurückführen. Da HDAC-Inhibitoren potentielle Medikamente für die Krebstherapie sind, könnte diese Isoenzymselektivität ein Vorteil für die klinische Anwendung von VPA als Krebsmedikament sein.

In dieser Studie wird gezeigt, dass PPAR δ -defiziente F9-Zellen und PPAR δ +/- ES-Zellen deutlich geringere Mengen an Histondeacetylase 2 (HDAC2) exprimieren, sowohl auf mRNA- wie auch auf Proteinebene. Überexpression von PPAR δ induziert HDAC2-Expression in F9-Zellen. Dabei kann es sich jedoch nicht um eine Liganden-induzierte Aktivierung des Rezeptors handeln, da der PPAR δ -Ligand pGI weder in F9-Zellen noch in der humanen colorektalen Krebszell-Linie HT-29 die HDAC2-Expression verstärkt. Demnach wird HDAC2 offensichtlich durch PPAR δ reguliert, wenn auch der exakte Mechanismus der Regulation unklar bleibt.

Histondeacetylasen spielen eine wichtige Rolle in der Transkriptionsregulation und in der Pathogenese bei Krebs. So ist zum Beispiel die aberrante Rekrutierung von HDAC-Aktivität entscheidend an der Pathogenese einiger Leukämieformen beteiligt. Die Regulation der HDAC2-Expression durch PPAR δ deutete darauf hin, dass HDAC2 eine Rolle bei der Pathogenese von Dickdarmkrebs spielen könnte. Die Expression von funktionellem Wildtyp-APC-Tumorsuppressorgen vermindert die HDAC2-Expression in HT-29-Zellen. Andererseits ist HDAC2 im Gewebe colorektaler Karzinome im Vergleich zu normalem Gewebe der gleichen Patienten überexprimiert. Wird die HDAC2-Expression durch RNAi-Technik unterdrückt, führt das zu einem

beträchtlichen Wachstumsarrest in HT-29-Zellen. Weitere Untersuchungen zeigten, dass diese Verringerung der HDAC2-Aktivität die Apoptose in HT-29-Zellen erheblich verstärkt, in geringerem Maße deren Differenzierung induziert, jedoch nicht den Verlauf des Zellzyklus beeinflusst. In Übereinstimmung mit diesen Beobachtungen antagonisiert die ektopische Expression von HDAC2 die APC-induzierte Apoptose in HT-29-Zellen. Insgesamt zeigen diese Beobachtungen, dass HDAC2 eine wichtige Rolle bei dem transformierten Phänotyp colorektaler Krebszellen spielt.

Zusammenfassend zeigt diese Studie, dass aberrante HDAC2-Expression, die durch PPAR δ reguliert sein könnte, für den transformierten und tumorigenen Phänotyp colorektaler Krebszellen wichtig ist. Darüber hinaus konnte gezeigt werden, dass VPA ein selektiver HDAC-Inhibitor ist, der präferenziell Klasse I-Enzyme hemmt und gleichzeitig HDAC2 selektiv für den Ubiquitin-Proteasom-vermittelten Proteinabbau markiert. Die angedeutete Rolle, die HDAC2 für die Karzinogenese spielen könnte, zusammen mit dem selektiven inhibitorischen Potential von VPA für Klasse I-HDACs, insbesondere HDAC2, deutet auf eine mögliche Anwendung von VPA bei der Therapie von Patienten mit Dickdarmkrebs hin.

Table of Contents

ABSTRACT	I
ZUSAMMENFASSUNG	III
TABLE OF CONTENTS	V
ABBREVIATIONS	VIII
1. INTRODUCTION	1
1.1 Valproic acid (VPA).....	1
1.1.1 Possible mechanisms of therapeutic effects of VPA	1
1.1.2 Teratogenicity of VPA.....	2
1.2 Peroxisome Proliferator-activated Receptor δ (PPAR δ).....	5
1.2.1 The family of PPARs	5
1.2.2 Evidence of the involvement of PPAR δ in VPA-induced teratogenicity	5
1.2.3 Structure and functional domains of PPAR δ	6
1.2.4 The expression pattern and ligands of PPAR δ	9
1.2.5 Biological functions of PPAR δ	9
1.3 Histone deacetylases (HDACs) and HDAC inhibitors.....	12
1.3.1 Histone acetylation and transcriptional regulation	12
1.3.2 The family of HDACs.....	14
1.3.3 Biological functions of HDACs	15
1.3.4 Involvement of HDACs in Tumorigenesis	16
1.3.5 HDAC inhibitors	18
1.3.6 Regulation of HDAC expression	22
1.4 The ubiquitin/proteasome system.....	23
1.5 Aims of this study.....	25
2. MATERIALS AND METHODS	26
2.1 Materials	26
2.1.1 Chemicals	26
2.1.2 Oligonucleotides.....	26
2.1.3 DNA probes for Southern and Northern blot analysis	27
2.1.4 Plasmids	27
2.1.5 Enzymes.....	28
2.1.6 Antibodies	28
2.1.7 Cell lines	29
2.2 General methods.....	31
2.2.1 Preparation of chemically competent <i>E. coli</i>	31
2.2.2 Transformation of <i>E. coli</i>	31
2.2.3 Small scale plasmid preparation from bacteria (Mini-prep)	32
2.2.4 Large scale plasmid preparation from bacteria (Maxi-prep)	32
2.2.5 Isolation and purification of DNA from agarose gel	33
2.2.6 Restriction endonuclease digestion of DNA	33
2.2.7 DNA ligation	33

2.2.8 Sub-cloning	33
2.2.9 Phenol/Chloroform extraction of nucleic acids	33
2.2.10 Determination of nucleic acid concentration	34
2.2.11 Isolation of genomic DNA from mammalian cells	34
2.2.12 Isolation of poly(A)+ RNA from mammalian cells	34
2.2.13 Polymerase chain reaction (PCR)	35
2.2.14 Cell culture	35
2.2.15 Stable and transient transfection of mammalian cells	37
2.2.16 Cell extracts preparation	38
2.2.17 Determination of protein concentration	39
2.2.18 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)	40
2.2.19 Coomassie staining	40
2.2.20 Radioactive labeling of DNA probes	40
2.2.21 Southern blot analysis	40
2.2.22 Northern blot analysis	41
2.2.23 Stripping DNA probes	41
2.2.24 Western blot analysis	42
2.2.25 Stripping Western blot membrane	42
2.2.26 Immunoprecipitation	43
2.3 Analytical Methods	43
2.3.1 Pulse-labeling and pulse-chase experiment	43
2.3.2 <i>In vivo</i> ubiquitination assay	44
2.3.3 Hoechst DNA staining	45
2.3.4 Flow cytometry (FACS)	45
2.3.5 Apoptosis assay	46
2.3.6 Immunohistochemistry (IHC)	46
2.3.7 <i>In vitro</i> histone deacetylase activity assay (HDAC assay)	47
2.3.8 <i>In vitro</i> histone acetyltransferase activity assay (HAT assay)	48
2.3.9 <i>In vitro</i> alkaline phosphatase activity assay (ALP assay)	48
3. RESULTS	49
3.1 Evidence that HDAC2 is regulated by PPARδ	49
3.1.1 Reduction of HDAC activity in PPAR δ -deficient F9 cells	50
3.1.2 Selective reduction of HDAC2 expression in PPAR δ -deficient F9 cells	51
3.1.3 Selective down-regulation of HDAC2 in PPAR δ +/- ES cells	53
3.1.4 Induction of HDAC2 upon PPAR δ overexpression	56
3.1.5 Unchanged HDAC2 expression upon ligand-activation of PPAR δ	56
3.1.6 Summary of this section	57
3.2 Roles of HDAC2 in transformed phenotype of colorectal cancer cells	59
3.2.1 Reduced HDAC2 expression upon restoring wt APC in HT-29 cells	61
3.2.2 Elevated HDAC2 expression in colorectal carcinoma tissues	64
3.2.3 Specific knock-down of HDAC2 by RNAi technology in HT-29 cell line	69
3.2.4 Reduction of HDAC2 leads to decreased cell numbers in HT-29 cell cultures	72
3.2.5 Reduction of HDAC2 does not affect the cell cycle progression of HT-29 cells	74
3.2.6 Reduction of HDAC2 increases apoptosis in HT-29 cells	76
3.2.7 HDAC2 reduction has a moderate effect on HT-29 cell differentiation	79
3.2.8 Ectopic HDAC2 expression antagonizes the APC-induced apoptosis	81
3.2.9 Summary of this section	81
3.3 VPA selectively inhibits a subset of HDAC activity	84
3.3.1 VPA inhibits N-CoR-associated HDAC activity <i>in vitro</i>	84
3.3.2 VPA inhibits HDAC2 activity <i>in vitro</i>	86
3.3.3 A VPA-insensitive HDAC subset is abundant in F9 cells	86
3.3.4 VPA preferentially inhibits class I HDACs	89
3.3.5 Summary of this section	91

3.4 VPA selectively accelerates ubiquitin/proteasome-mediated HDAC2 degradation ..	92
3.4.1 VPA, but not TSA, selectively reduces HDAC2 protein levels	92
3.4.2 VPA does not reduce the mRNA level of HDAC2	94
3.4.3 VPA accelerates HDAC2 protein degradation	96
3.4.4 VPA elevates the ubiquitination of HDAC2 protein	99
3.4.5 Summary of this section	99
4. DISCUSSION.....	101
4.1 VPA selectively targets class I HDAC, in particular HDAC2	101
4.1.1 VPA selectively inhibits class I HDAC activities	101
4.1.2 VPA selectively reduces HDAC2 protein level	101
4.1.3 Mechanisms of VPA-induced HDAC2 down-regulation	102
4.1.4 Anti-cancer effect of VPA	103
4.1.5 Correlation of HDAC inhibition and teratogenic activity of VPA-related compounds	104
4.2 Role of HDAC2 in colorectal cancer.....	104
4.2.1 Aberrant HDAC2 expression is essential for the transformed phenotype of HT- 29 cells	104
4.2.2 Questions to be answered: <i>in vivo</i> role, general oncogenic role of HDAC2 and HDAC2-regulated molecule(s)?	106
4.2.3 Perspectives of clinical relevance of HDAC2	107
4.3 PPAR δ	107
4.3.1 PPAR δ could be a regulator of HDAC2 expression	107
4.3.2 Mechanism of VPA-induced activation of PPAR δ -dependent transcription	108
4.4 Summary of the entire project.....	110
5. REFERENCES	111

Abbreviations

A	adenosine
aa	amino acid
ALP	alkaline phosphatase
AMP	acute myeloid leukemia
AP-2	activation protein 2
APC	adenomatous polyposis coli
APL	acute promyelocytic leukemia
bp(s)	base pair(s)
BSA	bovine serum albumin
C	cytidine
°C	degrees Celsius
CBP	CREB-binding protein
cDNA	complementary DNA
Ci	Curie
CLIM	cofactor of LIM homeodomain proteins
cm	centimeter
coREST	corepressor of REST
cPGI	carboprostaglandin
cPGI ₂	carbocyclic prostaglandin I ₂
cpm	count per minute
Cys	cystine
d	day
Da	Dalton
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E(10.5)	embryonic day (10.5)
ECL	enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>

EDTA	ethylenediamine-N,N-tetracetate
e.g.	example given
ELISA	enzyme linked immunoabsorbant assay
ES (cell)	embryonic stem (cell)
<i>et al.</i>	and others (Latin <i>et ali</i>)
FACS	fluorescence activated cell sorting
f.c.	final concentration
FCS	fetal calf serum
g	gram
G	guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GI	gastrointestinal
Glu	glutamine
GSK3-β	glycogen synthase kinase 3-β
³ H	tritium
HAT	histone acetyltransferase
HDA1	yeast histone deacetylase
HDAC	histone deacetylase
Hepes	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
His	histidine
hr(s)	hour(s)
HRP	horseradish peroxidase
HSB	high salt buffer
IC ₅₀	inhibitory concentration 50%
Ig	immunoglobulin
IHC	immunohistochemistry
IP	immunoprecipitation
KDa	kilodalton (10 ³ Daltons)
kb	kilo-base
L	liter
LBD	ligand-binding domain
m	milli- (10 ⁻³)

mA	milliamper
MEF-2	myocyte-specific enhancer binding factor 2
Met	methionine
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MyoD	myogenic factor
μ	micro- (10^{-6})
μ Ci	microcurie
μ g	microgram
μ l	microliter
μ M	micromolar
n	nano- (10^{-9})
N-CoR	nuclear receptor corepressor
ng	nanogram
Ni ²⁺ -NTA	nickel nitrilotriacetic acid
nm	nanometer
NR	nuclear receptor
nt	nucleotide
OD	optical density
p	pico- (10^{-12})
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
p-NPP	p-nitrophenylphosphate
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
pRB	retinoblastoma protein
REST	RE1 silencing transcription factor/neural restrictive silencing factor

RLIM	Ring finger LIM domain-binding protein
RNA	ribonucleic acid
RNAi	RNA-mediated interference
RPD3	histone deacetylase, originally defined as a mutation causing <u>R</u> educed <u>P</u> otassium <u>D</u> ependency in yeast
rpm	revolutions per minute
RT	room temperature
RXR	9- <i>cis</i> retinoic acid receptor
SDS	sodium-lauryl-sulfate
sec	second(s)
Sir2	silence information regulator 2
siRNA	small interfering RNA
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SRC	steroid receptor coactivator
T	thymidine
Tcf	T cell factor
Tris	Tris-(hydroxymethyl)-aminomethane
TSA	trichostatin A
U	uridine
Ubc	ubiquitin-conjugating enzyme
UV	ultraviolet
V	Volt
VPA	valproic acid
v/v	volume per volume
YY1	Yin-yang 1

1. Introduction

The work for this dissertation is based on previous findings that the anti-epileptic drug valproic acid (VPA) has teratogenic side effects, VPA induces differentiation of F9 teratocarcinoma cells, and VPA and its teratogenic derivatives selectively activate Peroxisome Proliferator-activated Receptor δ (PPAR δ) dependent transcription. Initially open questions of my thesis work were what role PPAR δ plays in VPA-induced teratogenicity and how VPA activates PPAR δ -dependent transcription. During the process of the studies, histone deacetylases (HDACs) were found to be modulated by VPA, e.g. VPA inhibits class I HDACs and down-regulates HDAC2 protein. In addition, PPAR δ was found to regulate HDAC2 gene expression under certain conditions. Based on these findings and the proposed positive role of PPAR δ in colorectal tumorigenesis (He *et al.*, 1999), question was defined on the role of HDACs in colorectal tumorigenesis. To provide the background knowledge for my study, this chapter presents an introduction to the effects of VPA; the structure and functions of PPAR δ ; HDACs and HDAC inhibitors; and the ubiquitin/proteasome pathway. Finally the aims of my study will be defined.

1.1 Valproic acid (VPA)

VPA, or 2-propylpentanoic acid, has been shown to possess multiple effects. It has been widely used for treating and preventing certain types of epileptic seizures for over twenty years (Ehlers *et al.*, 1992), and is also used as mood stabilizer in bipolar affective disorders (Muller-Oerlinghausen *et al.*, 2002). VPA is also known to have side effects during clinical treatment, e.g. teratogenicity.

1.1.1 Possible mechanisms of therapeutic effects of VPA

The exact mechanisms underlying the therapeutic actions of VPA are not well understood. One possible mechanism for the anti-epileptic effect of VPA could

be that VPA prevents the stimulation of nerves by increasing the concentrations in the brain of the neurotransmitter, gamma-aminobutyric acid (GABA) (Johannessen *et al.*, 2000). For VPA and other drugs in the therapy of bipolar disorders, the common molecular bases are assumed to be depletion of inositol and inhibition of the collapse of sensory neuron growth cones (Williams *et al.*, 2002). VPA probably act through more than one target due to the efficacy in diverse forms of epilepsy and bipolar disorder.

1.1.2 Teratogenicity of VPA

During the clinical treatment, VPA shows one major adverse effect, e.g. teratogenicity (reviewed in Nau *et al.*, 1991). With a probability around 1-2% women treated with VPA during early pregnancy give birth to newborns with spina bifida aperta, a severe neural tube closure defect. In addition, numerous other subtle malformations are observed, e.g. defects of the heart, the skeleton and the facial skull. These major and minors defects are collectively called the fetal valproate syndrome (DiLiberti *et al.*, 1984; Huot *et al.*, 1987; Ardinger *et al.*, 1988; Martinez-Frias, 1991). The fetal neural tube closure defects were also observed in mice upon proper VPA-treatment (Nau *et al.*, 1991; Ehlers *et al.*, 1992). Although in humans VPA appears to specifically induce posterior neural tube defects (NTDs), e.g. spina bifida but not anterior NTDs, the appropriate administration regimens of VPA in mice result in both posterior (spina bifida aperta and spina bifida occulta) and anterior (exencephaly) NTDs. Administration of VPA on day 8 of gestation in mice causes high rates of exencephaly, whereas administration of three consecutive high (450 mg/kg) or low (300 mg/kg) doses of VPA on day 9 of gestation produces a significant rate of spina bifida aperta or spina bifida occulta, respectively (Nau *et al.*, 1991).

Studies with VPA derivatives (structure shown in Fig. 1.1) indicated that the teratogenic potency depends strictly on the structure of the compound. Introduction of a double or triple bond between carbon 4 and 5 (4-en-VPA, 4-yn-VPA) increases teratogenicity whereas a double bond between carbon 2 and 3 (2-en-VPA) renders the derivative virtually non-teratogenic (Nau and

Löscher, 1986; Nau *et al.*, 1991; Bojic *et al.*, 1996). Interestingly, the teratogenic effect of VPA-related compounds is stereospecific. For instance, the (S)-enantiomer of 4-yn-VPA is highly teratogenic in mice while the (R)-enantiomer shows little or no effect (Nau *et al.*, 1991). Similarly, only one of the enantiomer of sodium 2-ethylhexanoate that is chemically related to VPA is teratogenic (Collins *et al.*, 1992). More structure-activity relationship studies demonstrated that the teratogenic properties of VPA derivatives do not correlate with antiepileptic activity, suggesting that the intended therapeutic activity in prevention of seizures requires mechanism(s) distinct from that underlying the teratogenic adverse effects (Nau *et al.*, 1991).

The molecular mechanisms by which VPA acts as a teratogen were largely unknown before this study although alterations in zinc and folate metabolism have been discussed (Löscher, 1999). The stringent structural constraints and stereo-selectivity with respect to teratogenicity suggest that possibly the cellular target molecules exist which specifically recognize VPA and its teratogenic derivatives (or metabolites thereof). Consequently functions of VPA-targeted molecules are altered. This in turn may result in an alteration of cellular process in the developing embryo leading to the birth defects.

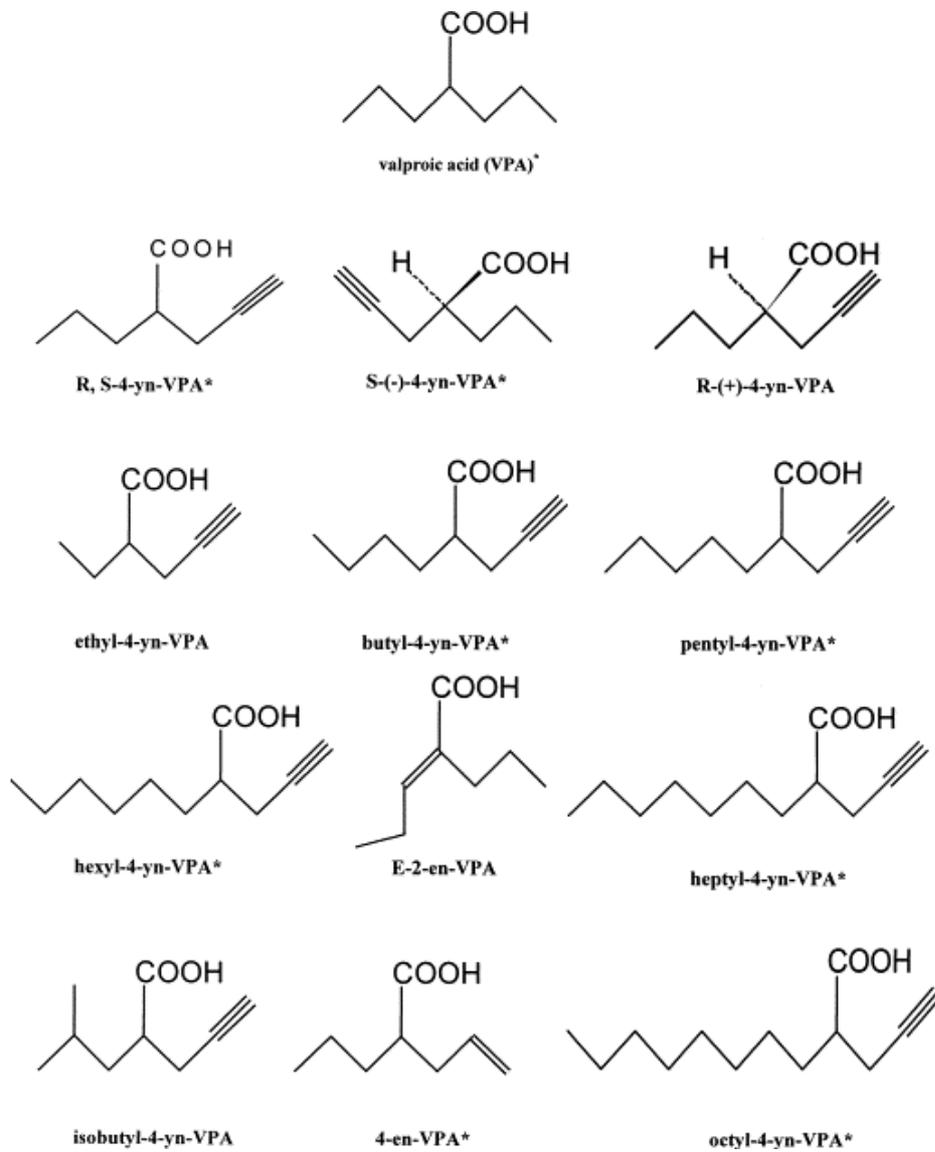


Figure 1.1 Chemical structure of VPA and derivatives. The teratogenic derivatives are indicated by asterisks (Lampen *et al.*, 2001).

1.2 Peroxisome Proliferator-activated Receptor δ (PPAR δ)

1.2.1 The family of PPARs

During the search for mediators of VPA teratogenicity, attention had been drawn to the peroxisome proliferator-activated receptors (PPARs) since VPA induces proliferation of peroxisomes in the rodent liver (Horie and Suga, 1985; Ponchaut *et al.*, 1991). PPARs belong to a subfamily of the nuclear hormone receptor (NR) superfamily. The NR superfamily comprises a large number of ligand-modulated transcription factors, including the classical steroid and thyroid hormone receptors (TRs), the retinoid hormone receptors, as well as many "orphan" receptors, e.g. family members with no physiological ligands or activators identified so far (Mangelsdorf *et al.*, 1995; Mangelsdorf and Evans, 1995). In general, NRs regulate the transcription of target genes by binding specific DNA sequences, so-called response elements, located in promoters of these genes. According to a unified nomenclature of NRs (Nuclear Receptors Nomenclature Committee, 1999), PPARs form the group C in the subfamily 1 of the NR superfamily. To date, there are three known PPAR isoforms, namely PPAR α (NR1C1), PPAR δ (also termed PPAR β , NUC-1 or FAAR) (NR1C2), and PPAR γ (NR1C3), which have been found so far in various species including rodent and human (Schmidt *et al.*, 1992; Sher *et al.*, 1993; Kliewer *et al.*, 1994).

1.2.2 Evidence of the involvement of PPAR δ in VPA-induced teratogenicity

The previous work in our lab and other groups suggests that PPAR δ could be involved in the teratogenicity of VPA. Only PPAR δ was found to be selectively activated by teratogenic derivatives of VPA, whereas PPAR α and PPAR γ were activated by VPA and some derivatives without discrimination between teratogenic and non-teratogenic compounds (Lampen *et al.*, 1999; 2001). In addition, only PPAR δ is expressed during the VPA-sensitive time of mouse

embryogenesis (Werling *et al.*, 2001). The F9 teratocarcinoma cell line was used as an *in vitro* cell differentiation model for monitoring the teratogenicity of VPA. VPA and its teratogenic derivatives induce a specific type of F9 cell differentiation, and knock-down of PPAR δ by antisense RNA expression precludes the response of F9 cells to VPA, suggesting a key role of PPAR δ in VPA-induced F9 cell differentiation (Werling *et al.*, 2001).

1.2.3 Structure and functional domains of PPAR δ

PPAR δ possesses the common structure of NR superfamily, namely, four major domains: activation function-1 (AF-1), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD) containing activation function-2 (AF-2) (Fig. 1.2, reviewed in Desvergne and Wahli 1999; Escher and Wahli, 2000). The function of the poorly conserved AF-1 domain of PPAR δ is not clear, while the corresponding domain of PPAR α and γ contributes to the ligand-independent regulation of the receptor activity upon AF-1 domain phosphorylation or an interdomainal regulation between AF-1 and LBD (Hu *et al.*, 1996; Shao *et al.*, 1998; Juge-Aubry *et al.*, 1999). The DBD is highly conserved among the NRs, which forms two “zinc finger” like motifs folded in a globular structure. The C-terminus of the first zinc finger comprises the so-called P-box that specifically bounds certain response elements (Schwabe *et al.*, 1993). The primary sequence of the PPAR δ P-box (CEGCKG) is identical to that of other subfamily 1 members, which all recognize the consensus AGGTCA (Mangelsdorf and Evans, 1995; Rastinejad *et al.*, 1995). The D-box (the region between the first two cysteine residues of the second zinc finger) is involved in DBD dimerization and recognition of the spacing between response element half-sites. PPAR δ typically forms a heterodimer with another member of the NR superfamily, the 9-*cis* retinoic acid receptor (RXR). PPAR δ in the heterodimer binds to PPAR response elements (PPRE) which are arranged with RXR response element in a direct repeat spaced by one nucleotide (DR1) (Fig. 1.3). An isoform-specific PPAR δ -responsive element was identified as CGCTCA-C (He *et al.*, 1999). The LBD of PPAR δ has been well studied with X-ray crystallography in the absence and in the presence of

ligands, revealing the structural determinants for ligands binding, ligand selectivity and heterodimerization (Xu *et al.*, 1999; Xu *et al.*, 2001). A model for ligand-modulated activation and repression of PPAR δ as well as some other NRs has been proposed based on the biochemical and structural studies. In the absence of ligand, the LBD of PPAR δ associates with corepressors such as N-CoR containing histone deacetylase (HDAC) activity that causes chromatin condensation and consequent repression of target gene expression (Shi *et al.*, 2002; Krogsdam *et al.*, 2002). Ligand-binding leads to conformational changes of the LBD, allowing release of corepressors and recruitment of coactivators (such as SRC-1) harboring histone acetyltransferase (HAT). HAT activity results in a relaxed chromatin structure that is thought to be required for the transcriptional activation (refer to section 1.3.1; Glass and Rosenfeld, 2000; Escher and Wahli, 2000) (Fig.1.3).

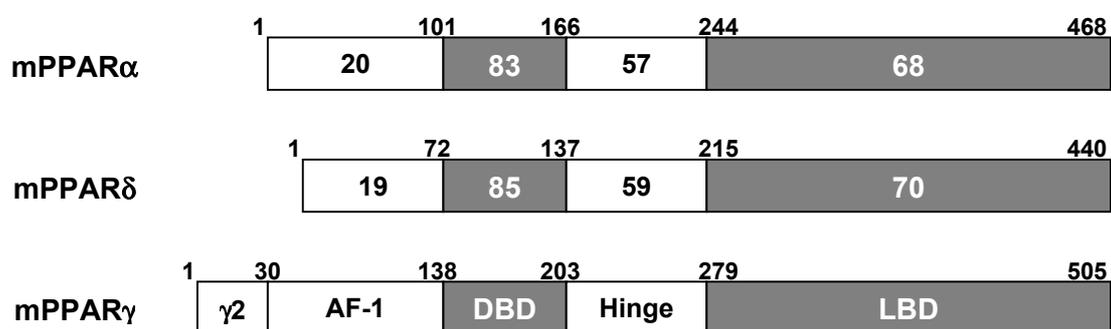
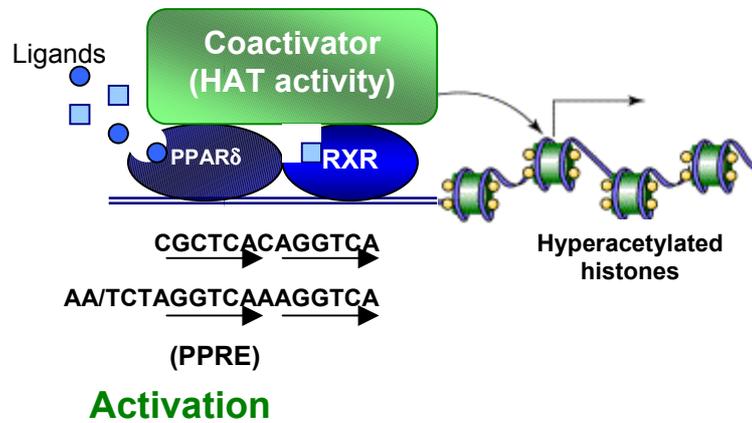


Figure 1.2 Structural and functional domains of PPARs. Mouse PPARs are shown. Numbers above schematic structures represent the linear position of amino acid residues from N-terminus of the proteins. Sequence similarities between isoforms are indicated by the numbers in the boxes. γ 2: PPAR γ 2-specific N-terminus of AF-1 domain that PPAR γ 1 and γ 3 do not contain; AF-1: activation function 1; DBD: DNA binding domain; Hinge: hinge region; LBD: ligand binding domain containing AF-2 (modified from Escher and Wahli, 2000).



Repression

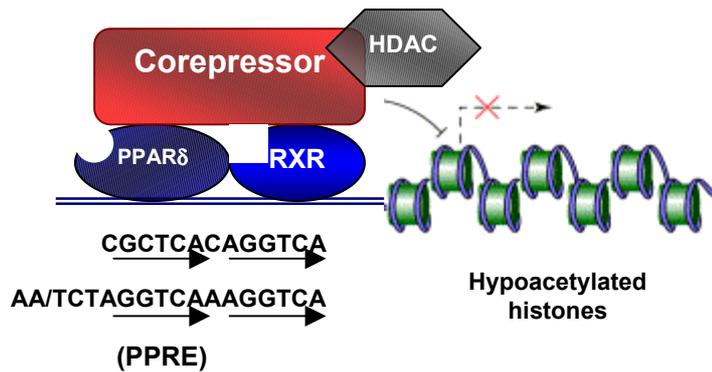


Figure 1.3 Model of PPAR δ transactivation and repression. The PPAR/RXR heterodimer binds to a PPRE (PPAR-response elements) located in the promoter of target genes through the DBDs of PPAR and RXR. In the absence of ligand, PPAR/RXR associated with corepressor/histone deacetylase (HDAC) complexes that cause hypoacetylation of histones and chromatin condensation, and in turn repress the transcription. In the presence of ligands, the activated PPAR/RXR heterodimer releases corepressor complexes and recruits cofactors containing histone acetyltransferase (HAT) activity, relaxing chromatin structure and consequently activating transcription of target genes.

1.2.4 The expression pattern and ligands of PPAR δ

PPAR δ expression begins at E9.5 during mouse embryogenesis as shown by Northern blot analysis (Kliwer *et al.*, 1994) or even earlier as detected by *in situ* hybridization (Werling *et al.*, 2001). In both rodent and human adults PPAR δ is ubiquitously expressed in most tissues detected (reviewed in Escher and Wahli, 2000).

Some naturally occurring fatty acids and eicosanoids as well as some synthetic compounds act as *bona fide* PPAR δ -binding ligands (Lehmann *et al.*, 1997; Kliwer *et al.*, 1997). Notably, the carbocyclic prostaglandin I₂ (cPGI₂) or iloprost was found to activate preferentially PPAR δ rather than the other two isoforms (Forman *et al.*, 1997).

1.2.5 Biological functions of PPAR δ

Functions of PPAR δ , which actually are poorly understood compare to the other two PPAR isoforms, are described below in detail since we have evidence that PPAR δ is involved in VPA-mediated teratogenicity (see above). Like the other two isoforms, PPAR δ is also possibly implicated in lipid metabolism (Leibowitz *et al.*, 2000), adipogenesis (Bastie *et al.*, 1999; Peters *et al.*, 2000), reverse cholesterol transport (Oliver *et al.*, 2001) and brain functions (Xing *et al.*, 1995; Peters *et al.*, 2000). Some specific roles of PPAR δ are emerging more recently in a number of physiological and pathological processes.

1.2.5.1 Embryo implantation

Lim *et al.* (1999) provided the evidence that activation of PPAR δ by cPGI₂ is essential for cyclo-oxygenase-2 (COX-2) mediated embryo implantation. Using COX-2 knockout mice, they suggested that the COX-2-derived prostacyclin cPGI₂ was the essential prostaglandin for implantation and

decidualization, and the effects of cPGI₂ were mediated by activating PPAR δ . However, PPAR δ is not required for embryo implantation in a knockout mouse model (Barak *et al.*, 2002). This finding suggests that PPAR δ might be not the exclusive molecular target of cPGI₂ in this process. On the other hand, if unliganded PPAR δ actively represses implantation and cPGI₂ relieves the inhibition, loss of PPAR δ could have no influence to the process of implantation.

1.2.5.2 Colorectal tumorigenesis

An interesting and important role of PPAR δ is its involvement in colon cancer (He *et al.*, 1999). Inactivating mutations of the *adenomatous polyposis coli* (APC) tumor suppressor gene are thought to be an initiating event for most colorectal cancers (Powell *et al.*, 1992; Kinzler and Vogelstein, 1996). One ability of wild-type (wt) APC relevant to its tumor-suppressive effects is binding to and promoting the degradation of β -catenin (Munemitsu *et al.*, 1995). β -catenin was initially discovered as an E-cadherin-binding protein, it was also shown later to be a component of the Wnt/wingless pathway (Morin, 1999; Polakis, 2000). In this pathway, β -catenin could act as a transcriptional cofactor via the association with T cell factor (Tcf) transcription factors, controlling the transcription of growth-promoting genes implicated in cell transformation, e.g. c-myc (Molenaar *et al.*, 1996; Behrens *et al.*, 1996; He *et al.*, 1998). Degradation of β -catenin protein by the ubiquitin/proteasome pathway (refer to 1.4) is crucial for regulation of β -catenin levels and subsequent β -catenin/Tcf signaling. Phosphorylation of β -catenin by serine-threonine kinase GSK3- β at specific sites is thought to elevate proteasomal degradation of β -catenin. APC assists the association of β -catenin and GSK3- β , hence increases β -catenin degradation and reduces the expression of β -catenin/Tcf-targeted genes, e.g. c-myc (Morin, 1997). In colorectal cancer cells, mutations of APC or activating mutations of β -catenin reduce the degradation of β -catenin. Accumulated cytoplasmic β -catenin protein in turn translocates into the nucleus and binds to Tcf transcription factors, resulting in

an increase of β -catenin/Tcf-mediated transcription (Morin *et al.*, 1997; Korinek *et al.*, 1997). Besides c-myc, cyclin D1 and some other genes, PPAR δ was identified as a novel downstream target gene of the APC/ β -catenin pathway (He *et al.*, 1999). PPAR δ expression is elevated in colorectal cancer possibly due to the increased β -catenin/Tcf signaling. Restoration of wt APC represses PPAR δ expression in colon cancer cell lines, suggesting a positive role of PPAR δ in colorectal tumorigenesis (He *et al.*, 1999). In addition, the PPAR δ null colon cancer cell line created by targeted homologous recombination exhibited a decreased ability to form tumors via being inoculated as xenografts in nude mice, suggesting that suppression of PPAR δ expression contributes to the growth-inhibitory effects of APC (Park *et al.*, 2001). It was also proposed that non-steroidal anti-inflammatory drugs (NSAIDs) such as sulindac inhibit tumorigenesis through antagonizing PPAR δ activity by the disruption of PPAR δ DNA binding, suggesting PPAR δ could serve as a therapeutic target in colon cancer (He *et al.*, 1999).

On the other hand, germ-line mutation of PPAR δ has only a moderate effect on the colon polyp formation in a mouse model with the mutated *APC* gene (*APC*^{min}), arguing against the essential role of PPAR δ in the process of colon polyp development (Barak *et al.*, 2002). A limitation in this study is the low survival of PPAR δ deficient mice during embryogenesis, which leaves the possibility that only those mice are accessible for analysis in which the loss of PPAR δ has been compensated for.

1.2.5.3 Epidermal wound healing

PPAR δ is proposed to be crucial for keratinocyte differentiation in wounded skin. In PPAR δ heterozygous mutant mice skin wound healing was found to be impaired, suggesting a role of PPAR δ in keratinocyte proliferation and/or differentiation in injured skin (Michalik *et al.*, 2001). A further study showed that keratinocyte differentiation requires the activation of PPAR δ . Activated

PPAR δ accelerates the differentiation of keratinocytes and increases their resistance to apoptotic signals (Tan *et al.*, 2001).

1.3 Histone deacetylases (HDACs) and HDAC inhibitors

In the process of revealing the mechanism by which VPA activates PPAR δ -dependent transcription, the attempt failed to show binding of VPA to PPAR δ (Göttlicher, personal communication; Lampen *et al.*, 2001). This observation raised the possibility that VPA activates the receptor by a mechanism distinct from direct binding as a ligand. Based on the models of activation (depression) of PPARs (refer to 1.2.3 and 1.3.1), the alternative mechanism could be that VPA, like some other short chain fatty acids, activates PPAR δ -dependent gene expression by inhibiting HDAC activity associated with the transcriptional repressor function of the receptor.

1.3.1 Histone acetylation and transcriptional regulation

Nucleosomes, the fundamental packing units of chromatin, are assembled by histone octamers (H2A, H2B, H3 and H4) and about 146 bps DNA are wrapped around each core histone octamer. Covalent modifications of the N-termini of the core histones in nucleosomes have important roles in chromatin remodeling and consequent transcriptional regulation. Known modifications consist of acetylation, phosphorylation, methylation and ubiquitination. Among them histone acetylation is well-characterized and appears to be the most dynamic one. Histone acetylation was already proposed to be associated with transcriptional activity in the 1960's (Allfrey *et al.*, 1964; Pogo *et al.*, 1966). The status of acetylation is regulated by two groups of enzymes, HATs and HDACs. HATs transfer acetyl group to the lysine residues on the histone N-terminal tails, thereby reducing the positive charge of histones and decreasing their affinity for DNA. Consequently, histone hyperacetylation is thought to lead to a relaxed chromatin structure that allows the accessibility of general transcriptional machinery, and in turn activates transcription. HDACs reverse this process, resulting in a subsequent condensation of chromatin and

ultimately transcriptional repression (reviewed in Pazin and Kadonaga, 1997; Struhl, 1998). HDAC inhibitors such as trichostatin A (TSA) or butyrate block HDAC activity and thereby induce the accumulation of hyperacetylated histones, leading to de-repression or apparent activation of transcription.

Both HAT and HDAC activities can be recruited to target genes in complexes with sequence-specific transcription factors and their cofactors. NRs are the main examples for understanding the regulation of histone acetylation and subsequent transcriptional activity by transcription factors and cofactors (reviewed in Xu *et al.*, 1999; Glass and Rosenfeld, 2000). In the absence of ligands, some NRs interact with corepressors, e.g. N-CoR and SMRT, which generally form large protein complexes containing HDAC activity and thereby suppress transcription. Upon ligand-binding, conformational changes of LBDs of NRs (see above) allow the release of corepressor complexes and the association with coactivators, e.g. SRC-1 and p300/CBP. Coactivator exists in a complex harboring HAT activity (many coactivators possess intrinsic HAT activity). Therefore, the ligand-induced switch of NR activation and repression reflects the exchange of coactivator and corepressor complexes with antagonistic enzymatic activities (Fig. 1.3). Intriguingly, many other transcription factors such as Mad, YY1 and ETO have also been shown to recruit HDAC- or HAT-containing complexes, suggesting that recruitment of HDACs/HATs is a common mechanism of regulation of gene expression (Yang *et al.*, 1996; Laherty *et al.*, 1997, Lutterbach *et al.*, 1998; Gelmetti *et al.*, 1998).

Besides core histones, some transcription factors such as p53, GATA-1 and YY1 are also shown to be the substrates of HATs and HDACs and could consequently be acetylated or deacetylated. The acetylation status of these transcription factors appears to modulate their DNA-binding abilities, suggesting another possible mechanism for HDACs and HATs to regulate gene transcription (Gu *et al.*, 1997; Boyes *et al.*, 1998; Yao *et al.*, 2001).

1.3.2 The family of HDACs

To date, more than 12 mammalian HDACs have been identified, falling into three classes (Table 1.1). The class I HDACs, about 50 kDa in size, are yeast RPD3 homologs harboring one catalytic domain in the middle region of the proteins. Class II enzymes, which are larger in size (about 120 kDa), have high homology to yeast Hda1, containing the catalytic domain near the C-terminus. Specifically, the class II enzyme HDAC6 possesses two catalytic domains that are both required for the full function of this enzyme (Grozinger *et al.*, 1999). The class III HDACs are yeast Sir2-like proteins which have NAD⁺-dependent deacetylase activity. While class I deacetylases show a predominant localization in the cell nucleus, class II HDACs shuttle between cytoplasm and nucleus, which appears to be regulated by binding to 14-3-3 proteins (Wang *et al.*, 2000; Verdell *et al.*, 2000; Grozinger *et al.*, 2000).

Table 1.1 Known mammalian HDACs

Class	HDAC	Reference of cloning
Class I (yeast Rpd3-related)	HDAC1	Taunton <i>et al.</i> , 1996; Furukawa <i>et al.</i> , 1996
	HDAC2	Yang <i>et al.</i> , 1996
	HDAC3	Yang <i>et al.</i> , 1997; Emiliani <i>et al.</i> , 1998
	HDAC8	Hu <i>et al.</i> , 2000; Buggy <i>et al.</i> , 2000; Van den Wyngaert <i>et al.</i> , 2000
	HDAC11	Gao <i>et al.</i> , 2002
Class II (yeast Hda1-related)	HDAC4	Wang <i>et al.</i> , 1999; Miska <i>et al.</i> , 1999; Grozinger <i>et al.</i> , 1999; Fischle <i>et al.</i> , 1999
	HDAC5	Grozinger <i>et al.</i> , 1999; Fischle <i>et al.</i> , 1999
	HDAC6	Grozinger <i>et al.</i> , 1999
	HDAC7	Fischle <i>et al.</i> , 1999; Kao <i>et al.</i> , 2000
	HDAC9	Zhou <i>et al.</i> , 2001
	HDAC10	Kao <i>et al.</i> , 2002; Guardiola and Yao, 2002, Tong <i>et al.</i> , 2002
Class III (yeast Sir2-related)	SIRT1-7	Afshar and Murnane, 1999; Frye, 1999; Frye, 2000

The corepressors N-CoR, SMRT and Sin3 are bridging factors for the recruitment of HDACs to transcription factors. N-CoR can bind to most known class I and II HDACs, working like a “platform” for these enzymes (Heinzel *et al.*, 1997; Alland *et al.*, 1997; Nagy *et al.*, 1997; Huang *et al.*, 2000; Kao *et al.*, 2000, Wen *et al.*, 2000).

1.3.3 Biological functions of HDACs

At present only limited information is available for the biological functions of individual HDACs. Class I enzymes seem to be involved in more general cellular processes such as cell cycle regulation and cell differentiation. HDAC1 and 2 are involved in cell cycle regulation upon association with pRB and E2F and regulating the expression of cell cycle regulators (reviewed in Wang *et al.*, 2001). More recently the HDAC1 knockout mouse model provided the first *in vivo* evidence that a histone deacetylase is essential for unrestricted cell proliferation by repressing the expression of cell cycle inhibitors such as p21 and p27 (Lagger *et al.*, 2002). Class I enzymes are implicated in neuronal cell differentiation. HDAC1 and HDAC2 are required for silencing neural-specific genes upon being recruited by the transcription factor REST, which plays a key role in restricting neuronal traits in nonneuronal cells (Ballas *et al.*, 2001). HDAC1 is also involved in skeletal myogenesis. It has been shown that HDAC1 interacts with MyoD, an important transcription factor for muscle differentiation, in undifferentiated myoblasts and mediates repression of muscle-specific gene expression (Mal *et al.*, 2001; Puri *et al.*, 2001).

In contrast, class II HDACs might have tissue-specific functions during the execution of developmental programs. A large body of evidence has demonstrated that class II enzymes associate with MEF-2 transcription factor and in turn suppress smooth and skeletal muscle differentiation. MEF-2 is a transcription factor that controls the expression of muscle-specific genes. MEF-2 recruits class II HDACs in the cell nucleus and suppresses the expression of genes required for muscle differentiation. When myoblasts are triggered to differentiate, class II enzymes are released from MEF-2 and

shuttle from the nucleus to the cytoplasm (Miska *et al.*, 1999; Lu *et al.*, 2000; McKinsey *et al.*, 2000; Dressel *et al.*, 2001; Wu and Olson, 2002).

Sir2-like HDACs are structurally different from the other two classes. It has been reported that Sir2-like HDACs are involved in the negative regulation of p53 function via deacetylating p53. Consequently they could modulate cellular senescence and promote cell survival under stress (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002).

1.3.4 Involvement of HDACs in Tumorigenesis

Aberrant recruitment of HDAC as well as HAT activity is proposed to be associated with cancer development (reviewed in Krämer *et al.*, 2001; Marks *et al.*, 2001). HDACs are crucially involved in mediating the oncogenic function of translocation fusion proteins in certain leukemia and lymphoma. One classic example is acute promyelocytic leukemia (APL). The oncoproteins PML-RAR and PLZF-RAR, produced by chromosomal translocation between the genes for RAR α and either the promyelocytic leukemia protein (PML) or the promyelocytic zinc finger protein (PLZF), repress transcription by associating with corepressor complexes containing HDAC activity. Acute myeloid leukemia subtype M2 is associated with the t(8;21) translocation that generates the AML1/ETO fusion protein. AML1/ETO is a potent transcriptional repressor through recruitment of HDAC-containing complexes. In non-Hodgkin's lymphoma, inappropriate overexpression of the BCL6/LAZ3 transcription factor that interacts with corepressors/HDACs complexes leads to lymphoid oncogenic transformation. However, so far there is poor evidence for the involvement of aberrant regulation in the transcriptional corepressor/HDAC system in a non-hematopoietic form of cancer.

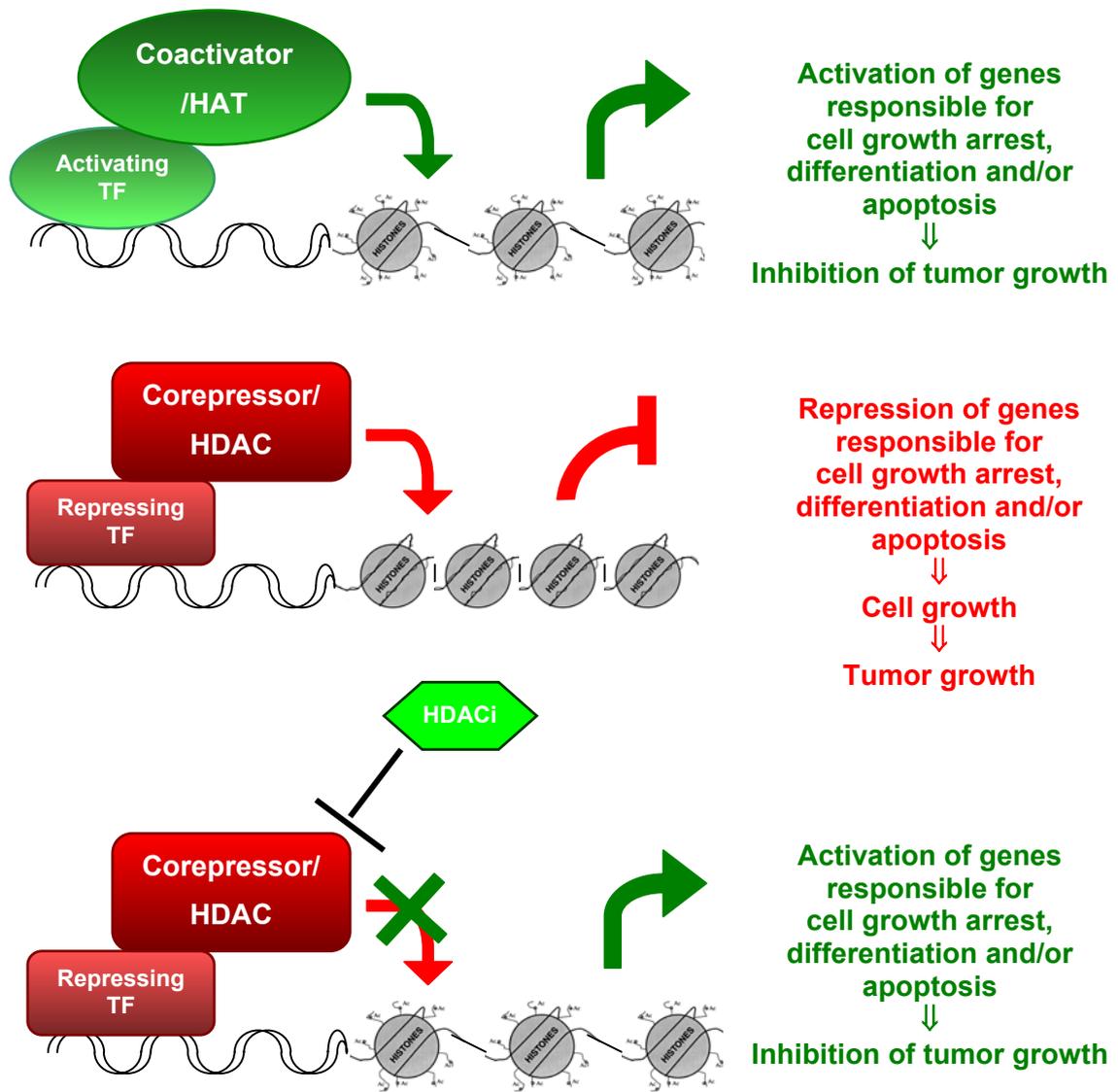


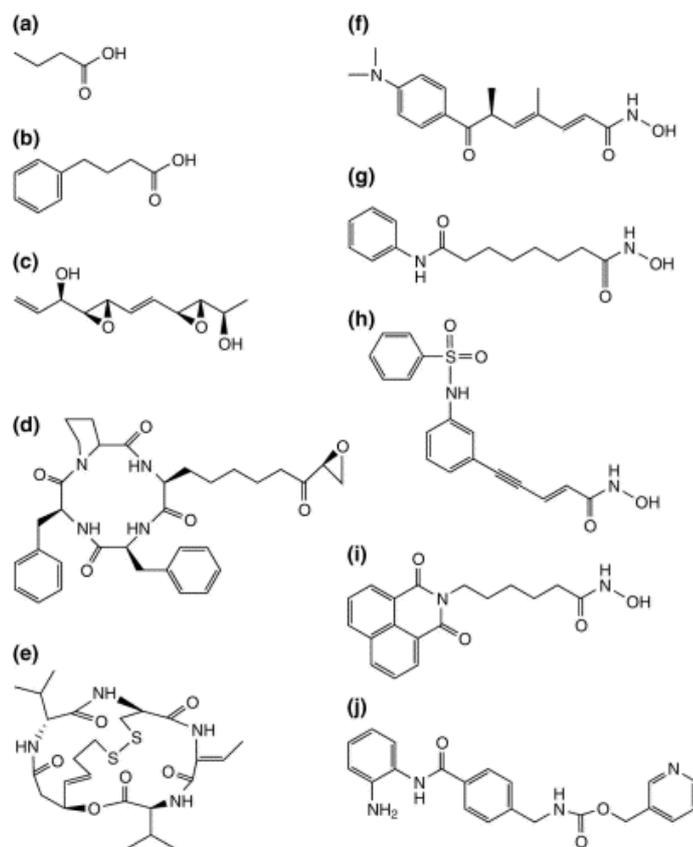
Figure 1.4 Impact of HDAC/HAT and HDAC inhibitors on transcription and tumorigenesis. See text for details. TF: transcription factor, HDACi: HDAC inhibitor.

In these examples, transcriptional repression appears to be mediated by the aberrant recruitment of HDACs, resulting in an imbalance in histone (or other protein) acetylation which possibly dysregulates genes involved in control of cell cycle progression, cell differentiation or apoptosis. Logically, HDAC inhibitors could be promising candidate drugs for the treatment of these kinds of cancer patients (Fig. 1.4).

1.3.5 HDAC inhibitors

Structurally, the HDAC inhibitors known so far can be classified into four groups (Fig. 1.5). The mechanism of the inhibitory activity of hydroxamic acid-related compounds has been proposed to be binding to the catalytic site of HDACs, thereby blocking substrate access of the enzymes, as shown by X-ray crystal structure studies (Finnin *et al.*, 1999). Competition with substrates for the catalytic center is also proposed as the mechanism by which other compounds inhibit HDAC activity. Among these HDAC inhibitors, butyrate was the first one to be identified (Boffa *et al.*, 1978). However, butyrate is far less potent than other compounds (see Fig. 1.5). In addition, butyrate shows the impact not only on HDACs, but also on enzymes conferring phosphorylation, protein methylation as well as DNA methylation (Archer and Hodin, 1999). Other HDAC inhibitors are more potent and more specific.

Both class I and II HDACs are inhibited by the known HDAC inhibitor trichostatin A (TSA) whereas class III HDACs are insensitive to TSA.



Structural class		Name of the inhibitor	Effective inhibitory concentration range	Clinical trials
Short chain fatty acids	A	Butyrate	mM	+
	B	Phenylbutyrate	mM	+
Cyclic tetrapeptides	D	Trapoxin	nM, irreversible binding	-
	E	Depsipeptide	μ M	+
Hydroxamic acids	F	Trichostatin A	μ M	-
	G	SAHA	μ M	+
	H	Scriptaid	μ M	-
	I	Oxamflatin	μ M	-
Epoxides Benzamides	C	Depudecin	μ M, irreversible binding	-
	J	MS-275	μ M	-

Figure 1.5 Chemical structure and other data of selected HDAC inhibitors (modified from Krämer *et al.*, 2001 and Marks *et al.*, 2001).

1.3.5.1 Genes that are regulated by HDAC inhibitors

Ample evidence shows that HDAC inhibitors induce hyperacetylation of core histones both *in vitro* and *in vivo*. Furthermore, they induce cell cycle arrest, cell differentiation and apoptosis in a plethora of cultured tumor cell lines. Since histone acetylation is associated with activation of gene transcription, the cellular effects of HDAC inhibitors is possibly due to regulation of a certain set of genes which are responsible for cell growth, differentiation and apoptosis. In a variety of cell lines studied, induction of p21^{WAF1} has been found most frequently after the treatment of HDAC inhibitors (reviewed in Krämer *et al.*, 2001; Marks *et al.*, 2001). p21^{WAF1} blocks cell cycle progression via inhibiting cyclin-dependent kinase activity and, as a consequence, causing cell cycle arrest in the G1 phase. Bcl-2 and Bcl-XL proteins that antagonize apoptosis are down-regulated by HDAC inhibitors. Regulation of some tumor suppressors and oncoproteins, such as c-myc, p53 and gelsolin, also appears in certain HDAC inhibitor-treated cancer cell lines. The regulation of these genes may contribute to HDAC inhibitor-induced cellular phenotypes.

Hyperacetylation was induced upon HDAC inhibitor treatment both in normal and tumor cells, however, the growth inhibition and enhanced apoptosis effects seemed to be confined to transformed cells (Parsons *et al.*, 1997; Qiu *et al.*, 1999; Kim *et al.*, 1999). In SAHA- or TSA-treated cells only about 2% of expressed genes showed a change in transcription patterns although the bulk histone hyperacetylation was observed, suggesting that only a small sub-population of genes is controlled by histone deacetylation (Van Lint *et al.*, 1996; Marks *et al.*, 2000). This regulation of a limited set of genes could be the reason why HDAC inhibitors preferentially affect tumor cells, rather than being toxic to the whole organism.

1.3.5.2 *In vitro* and *in vivo* experimental models for the effects of HDAC inhibitors

Many cultured transformed cell lines were found to be sensitive to HDAC inhibitors, including cells derived from hematological tumors (leukemias, lymphomas and myelomas) and from solid tumors such as breast, colon, bladder, prostate and ovarian tumor (reviewed in Marks *et al.*, 2000; Krämer *et al.*, 2001; Marks *et al.*, 2001). Broad range of tumor cells affected by HDAC inhibitors presents a promising perspective for anti-tumor activity in many different forms of cancer. However, in some cases it is unclear that these effects were due to gene expression regulation by HDAC inhibition or just a toxic effect inherent to the compounds applied.

Several *in vivo* models have also been established in tumor-bearing rodents (reviewed in Krämer *et al.*, 2001, Marks *et al.*, 2001). HDAC inhibitors significantly reduced the tumor growth and metastasis in experimental animals, showing no considerable adverse effects at doses that inhibit tumor growth remarkably. The inhibited tumor types cover leukemia and several solid tumors including colon, gastric, lung, prostate, breast and some others, suggesting a variety of tumor types that could possibly response to therapy with HDAC inhibitors.

TSA and depsipeptide also antagonize angiogenesis *in vitro* and *in vivo*, possibly by inhibiting HDACs that suppress hypoxia-responsive tumor suppressor genes (Kim *et al.*, 2001; Kwon *et al.*, 2002). This finding suggests a new concept that HDAC inhibitors could block tumor growth indirectly by inhibiting new blood vessel formation of tumors.

Besides anti-cancer effects, another potential application of HDAC inhibitors is antagonizing poly-glutamine-dependent toxicity in certain neurodegenerative diseases, as shown in a *Drosophila* model and a cell culture system (Steffan *et al.*, 2001; McCampbell *et al.*, 2001).

1.3.5.3 Clinical applications of HDAC inhibitors

A case report showed that a young APL (PML-RAR) patient with relapses after *all-trans* retinoic acid treatment (standard therapy for APL) had a complete remission for 7 months by treating with HDAC inhibitor phenylbutyrate (Warrell *et al.*, 1998). Currently, several HDAC inhibitors are in clinical trials, including SAHA (Phase I), depsipeptide (Phase I and II) and VPA (Phase I and II) (Göttlicher *et al.*, 2001 and results in this thesis). The drugs are administered to patients with hematological malignancies as well as those with various types of solid tumors, although some leukemias appeared more sensitive to certain HDAC inhibitors (reviewed in Marks *et al.*, 2001). Combination with other chemotherapies might be more efficient than monotherapy for at least some kinds of cancer patients (Minucci *et al.*, 2001; Marks *et al.*, 2001).

1.3.6 Regulation of HDAC expression

The regulation of HDAC expression is largely unclear. The regulation may occur both at transcriptional level and at post-transcriptional levels. HDACs 1, 2 and 3 were shown to be induced at mRNA levels upon treatment with TSA and butyrate in cultured cells (Gray and Ekstrom, 1998; Dangond and Gullans., 1998). The expression of HDAC1 was shown to be up-regulated by the growth factor IL-2 in murine T cells. More recently, it was suggested that HDAC1 could be down-regulated via proteasome-mediated protein degradation (Zhou *et al.*, 2000). Several post-translational modifications of HDACs have been discovered, such as phosphorylation of HDAC1 and 2, SUMOylation of HDAC1 and HDAC4 and ubiquitination of HDAC1 (Galasinski *et al.*, 2002; David *et al.*, 2002; Kirsh *et al.*, 2002). Ubiquitination is the most important covalent modification that makes proteins for degradation through the proteasome pathway.

1.4 The ubiquitin/proteasome system

The ubiquitin/proteasome pathway was discovered some 20 years ago and was originally thought to eliminate abnormal and damaged proteins (Hershko *et al.*, 2000). Now we know that this pathway is responsible for the highly selective proteolysis of intracellular proteins in general cellular environments. The ubiquitin/proteasome pathway was also found to play proteolysis-independent roles, e.g. protein kinase activation and subnuclear trafficking (reviewed in Pickart *et al.*, 2001). Degradation of a protein via the ubiquitin/proteasome pathway involves two successive steps: 1) conjugation of multiple ubiquitin moieties to the protein substrate and 2) targeted degradation of the ubiquitinated protein by the 26S proteasome complex. Conjugation of ubiquitin to the substrate proceeds through three sequential steps using three groups of enzymes. Firstly, ubiquitin, a 76 aa, highly conserved and ubiquitously expressed protein, is activated to a thioester by ubiquitin-activating enzyme (E1), then transferred to one of several ubiquitin-conjugating enzymes (Ubc or E2). Finally E2s collaborate with many protein-ubiquitin ligases (E3) that specifically bind to protein substrates, transferring ubiquitin to the ϵ -amino group of a lysine residue in the substrate to generate an isopeptide bond. After creating the first ubiquitin-conjugate, the polyubiquitinated protein is produced by progressive transfer of additional ubiquitins to Lys48 of the previously conjugated ubiquitin by the same cascade. The polyubiquitin chain serves as a recognition maker for the 26S proteasome, which contains two ATP containing 19S subunits for specifically recognizing polyubiquitin chains and one 20S proteasome for degrading protein substrates (Fig. 1.6). Most organisms have one E1, a significant but limited number of E2s, and many E3s (Ciechanover *et al.*, 2000).

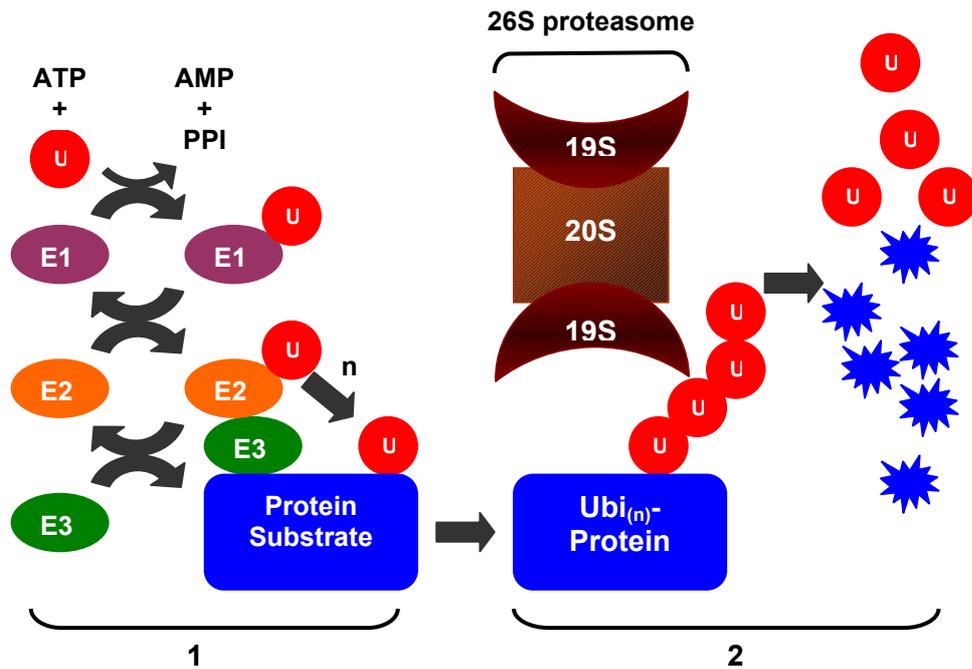


Figure 1.6 Mechanism of ubiquitin/proteasome-mediated protein degradation. Step 1: poly-ubiquitination of target protein; step 2: recognising and degrading ubiquitinated protein by 26S proteasome. See text for details of the procedures. U: ubiquitin, n: mono- to poly-.

1.5 Aims of this study

The aims of this study were based on the previous findings of the laboratory, e.g. that VPA induces differentiation of F9 teratocarcinoma cells, and that this differentiation-inducing activity is associated with activation of PPAR δ -dependent transcription by VPA.

1. Elucidation of the mechanism(s) underlying activation of transcription by VPA with focuses on PPAR δ and its cofactors.

Based on conclusions from this aim, e.g. that VPA inhibits histone deacetylases (HDACs), and the finding that PPAR δ is a downstream target gene of the APC/ β -catenin pathway (He *et al.*, 1999), the following aims were defined.

2. Investigating whether, and if so by which mechanism(s), VPA may alter HDAC expression in addition to inhibition of enzymatic activity.
3. Evaluation of the regulation of HDAC expression by PPAR δ and APC.
4. Determination of the role of HDAC2 for the transformed phenotype of colorectal cancer cells upon loss of functional APC.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All general chemicals were, unless otherwise stated, supplied by Carl Roth GmbH & Co (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Sigma Chemie GmbH (Deisenhofen, Germany) and were of the highest purity grade. Radioactive chemicals were purchased from Amersham Pharmacia biotech (Buckinghamshire, UK). Aqueous solutions were prepared with water purified by the Milli-Q plus water purification system (Millipore, Molsheim, Germany).

2.1.2 Oligonucleotides

2.1.2.1 DNA oligonucleotides

All DNA oligonucleotides were synthesized by MWG biotech GmbH (Ebersberg, Germany) with HPSF-purified grade.

Primers for PCR amplification of murine HDAC2 cDNA probe for Northern blot analysis:

HD2-probe-forw 5'- GCT ATT CCA GAG GAT GCT GTT C – 3'

HD2-probe-rev 5'- GTT GCT GAG TTG TTC TGA CTT G – 3'

2.1.2.2 RNA oligonucleotides (small interfering RNA)

All RNA oligonucleotide duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, USA) and were >97% pure with no contaminating single-strand RNA. The specificity of siRNA was proven by BLAST-search (NCBI program).

For RNAi experiment:

siRNA-HDAC2-1 duplex	5'- GCC UCA UAG AAU CCG CAU G dTdT – 3'
	3'- dTdT CGG AGU AUC UUA GGC GUA C – 5'
Luciferase GL2 duplex	5'- CGU ACG CGG AAU ACU UCG A dTdT –3'
	3'- dTdT GCA UGC GCC UUA UGA AGC U – 5'

2.1.3 DNA probes for Southern and Northern blot analysis

PPAR δ XX5.0 probe for Southern blot analysis of homologous recombination	567 bps XbaI-EcoRI fragment from the plasmid pmFAAR-XX5.0
PPAR δ probe for Northern blot analysis	1 kb EcoRI fragment from the plasmid pSG-FAAR, corresponding to nt 195-1194 of the murine PPAR δ coding region
HDAC2 probe for Northern blot analysis	312 bps PCR product (primer shown above) from the plasmid pCMV-mRPD3-2.1, corresponding to nt 1336-1647 of the murine HDAC2 coding region
GAPDH probe for Northern blot analysis	1.3 kb PstI fragment from the plasmid pGAPDH-13 (a gift from M. Litfin)

2.1.4 Plasmids

The names, inserts and sources of plasmids used in this study are listed.

Name	Insert/usage	Source
pcDNA3.1(+)	empty vector	Invitrogen, Karlsruhe, Germany

pCMV-mRPD3-2.1	Murine HDAC2 cDNA	Dr. Seto, Tampa, USA
pCMV-GFP	GFP cDNA	Dr. Göttlicher, Karlsruhe, Germany
pHis ₆ -Ubi(pMT107)	His ₆ -tagged Human ubiquitin (8x)	Dr. Bohmann, Heidelberg, Germany
pmFAAR-XX5.0	5 kb Xbal fragment of murine PPAR δ genomic DNA	Dr. Göttlicher, Karlsruhe, Germany
pPNT-FAAR-5-3	Targeting vector for PPAR δ knockout	Dr. Göttlicher, Karlsruhe, Germany
pSG-FAAR	Murine PPAR δ cDNA	Dr. Grimaldi, Nice, France
pUbi-FAAR	Murine PPAR δ cDNA	Dr. Göttlicher, Karlsruhe, Germany
pUbi-GFP	GFP cDNA	Dr. Göttlicher, Karlsruhe, Germany

2.1.5 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Invitrogen GmbH (Karlsruhe, Germany), Promega (Mannheim, Germany) or New England Biolabs (Beverly, USA) unless otherwise stated.

2.1.6 Antibodies

2.1.6.1 Primary antibodies

The names and sources of primary antibodies used in this study are listed.

Name	Source
Actin, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-1616
β -catenin, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-1496
HDAC1, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-6298
HDAC2, mouse monoclonal	Santa Cruz (Santa Cruz, USA), sc-9959
HDAC2, rabbit polyclonal	Zymed (San Francisco, USA), 51-5100
HDAC3, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-8138
HDAC5, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-5252
HDAC6, goat polyclonal	Santa Cruz (Santa Cruz, USA), sc-5253
His-tag, mouse monoclonal	Santa Cruz (Santa Cruz, USA), sc-8036
mSin3B, rabbit polyclonal	Santa Cruz (Santa Cruz, USA), Ak-12
N-CoR, rabbit polyclonal	a gift from Dr. T. Heinzel

2.1.6.2 Secondary antibodies

Secondary antibodies used in this study are listed.

Name	Supplier
HRP conjugated goat anti mouse IgG	DAKO (Glostrup, Denmark)
HRP conjugated goat anti rabbit IgG	DAKO (Glostrup, Denmark)
HRP conjugated rabbit anti goat IgG	DAKO (Glostrup, Denmark)

2.1.7 Cell lines

All media and other reagents for cell culture were purchased from Invitrogen GmbH (Karlsruhe, Germany). FCS was purchased from PAA laboratories GmbH (Linz, Austria). ES-cell grade FCS was supplied by Biochrom KG (Berlin, Germany). Cell lines used in this study are shown below.

Name	Origin	Source	Medium
ES (E14.1)	Mouse embryonic stem cells	Dr. Schorle, Bonn, Germany	DMEM (high glucose), 15% FCS (ES-cell grade), 2 mM L-glutamine, 100 μ M 2-mercaptoethanol, 1000 U/ml LIF, 1 \times non-essential aa 100U/ml/100 μ g/ml ampicillin/streptomycin
F9	Mouse embryonic teratocarcinoma cells	Dr. Göttlicher, Karlsruhe, Germany	DMEM/Ham's F-12 (1:1), 10% FCS, 2 mM L-glutamine, 100 μ M 2-mercaptoethanol
F9 PPAR δ antisense clones 6, 10 and 42	Mouse embryonic teratocarcinoma cells	Dr. Göttlicher, Karlsruhe, Germany	Same as above
HEK293T	Human embryonic kidney cells	Dr. Schneider, Karlsruhe, Germany	DMEM, 10% FCS, 2 mM L-glutamine
HT-29	Human colon carcinoma cells	Mr. Chen, Karlsruhe, Germany	DMEM, 10% FCS, 2 mM L-glutamine
HT-29-APC	Human colon carcinoma cells	Dr. Vogelstein, Baltimore, USA	McCoy 5A, 10% FCS, 0.6 mg/ml hygromycin B
HT-29- β GAL	Human colon carcinoma cells	Dr. Vogelstein, Baltimore, USA	Same as above

2.2 General methods

The majority of protocols and recipes for commonly used buffers in this study were taken from the laboratory manual of Sambrook *et al.* (1989) and Current Protocols in Molecular Biology (Ausubel *et al.*, 1989) unless otherwise stated.

2.2.1 Preparation of chemically competent *E. coli*

A single colony of *E. coli* DH5 α was incubated in 5 ml LB medium (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) overnight at 37°C with shaking (200 rpm). Then 4 ml was removed to 400 ml fresh LB medium and allowed to grow to an OD₅₉₀ of 0.375. After chilling on ice for 10 min, the cells were centrifuged at 3,600 g for 10 min at 4°C. The pellet was re-suspended in 20 ml of ice cold 0.1 M CaCl₂ and allowed to stand on ice for 10 min. After centrifugation, the pellet was re-suspend in a further 20 ml of ice-cold CaCl₂ and this process was repeated once more. Finally the pellet was re-suspended in 2 ml of ice-cold CaCl₂ with 10% glycerol. After 5 min incubation on ice, the bacteria were dispensed in 200 μ l aliquots and frozen down at –80°C.

2.2.2 Transformation of *E. coli*

Chemical transformation was used for propagation of plasmids and DNA ligation products. 1 μ l plasmids (0.01–1 μ g) or 1–2 μ l of a ligation mix was added to 50 μ l ice-thawed chemically competent *E. coli*. After mixture and incubation on ice for 30 min bacteria were heat-shocked at 42°C for 90 sec and incubated on ice for another 4 min. The transformed bacteria were mixed with 800 μ l SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM Mg₂SO₄ and 20 mM glucose) and incubated at 37°C with shaking for 45 min. Finally the bacteria were plated onto the LB agar dishes supplemented with appropriate antibiotics and allowed to grow for 16 – 24 hours at 37°C.

2.2.3 Small scale plasmid preparation from bacteria (Mini-prep)

2.2.3.1 Phenol-Chloroform method

Bacteria culture was pelleted by centrifugation at 14,000 rpm for 30 sec and re-suspended in residual 100 μ l LB medium. Then 50 μ l of phenol/chloroform (1:1) was added and vortexed. After centrifugation at 14,000 rpm for 5 min, the supernatant was mixed with equal amount of chloroform, again vortexed and centrifuged. The aqueous phase was taken to precipitate DNA with 2.5 volumes of pure ethanol and NaCl (f.c. 0.1 M) on ice for 30 min. DNA was collected by centrifugation at 14,000 rpm for 10 min. Following washing in 70% ethanol, the DNA pellet was air-dried and dissolved in 50 μ l TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

2.2.3.2 Non-Phenol-Chloroform method (modified from Qiagen Plasmid Maxi Kit)

Bacteria were pelleted by centrifugation at 14,000 rpm for 30 sec and re-suspended in 150 μ l of P1 buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8 and 0.4 mg/ml RNase A). The cells were then lysed by addition of 300 μ l of P2 buffer (200 mM NaOH and 1% SDS), and subsequently gently mixed with 225 μ l P3 buffer (3 M potassium acetate, pH 5.5). After incubating on ice for 20 min and centrifugation at 14,000 rpm for 10 min, the plasmid DNA-containing supernatant was removed, and the DNA was precipitated with addition of 2.5 volumes of pure ethanol. After pelleting the DNA by centrifugation at 14,000 rpm for 10 min, it was washed with 70% ethanol. The plasmid DNA was finally dissolved in 50 μ l TE buffer.

2.2.4 Large scale plasmid preparation from bacteria (Maxi-prep)

A 200-ml overnight culture of *E. coli* transformed with an appropriate plasmid was used for the Maxi-preparation. The Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions.

2.2.5 Isolation and purification of DNA from agarose gel

The EasyPure DNA purification Kit (Biozyme, Oldendorf, Germany) was used to isolate the appropriate DNA fragments in the agarose gel (e.g. restriction-digested vectors and PCR products), following the instruction of the manufacturer.

2.2.6 Restriction endonuclease digestion of DNA

Usually 1-2 or 2-3 units of a restriction enzyme were used for each μg plasmid DNA or genomic DNA, respectively. The reactions were carried out according to the instructions of the suppliers. The quality of the digest was controlled by the gel electrophoresis.

2.2.7 DNA ligation

All DNA ligation reactions were performed in a total volume of 10 μl and incubated overnight at 15°C, using the ligases and buffers supplied by the manufacturer (Invitrogen, Karlsruhe, Germany)

2.2.8 Sub-cloning

The DNA insert was released from the vector by using appropriate restriction enzymes, purified by a gel electrophoresis and EasyPure Kit (Biozyme, Oldendorf, Germany), and subsequently cloned into the new vector by the compatible sites or blunt ends ligation.

2.2.9 Phenol/Chloroform extraction of nucleic acids

To remove protein contaminants from nucleic acid, an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added and vortexed. After centrifugation at 14,000 rpm for 10 min, the upper aqueous phase containing

nucleic acid was carefully transferred to a fresh micro-tube and subjected to another round of extraction with chloroform/isoamylalcohol (24:1).

2.2.10 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by spectroscopic measurement of their optical density (OD) at 260 nm and 280 nm. An OD value of 1 at 260 nm is equivalent to 50 µg/ml of double stranded DNA, 40 µg/ml RNA or 20 µg/ml single-stranded oligonucleotide. Pure DNA and RNA in aqueous solution should have an OD_{260}/OD_{280} ratio of 1.6-1.8 and 1.8-2.0, respectively.

2.2.11 Isolation of genomic DNA from mammalian cells

Cells were washed with Ca/Mg free PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) and lysed in 500 µl genomic DNA extraction buffer (50 mM Tris-HCl pH 8, 100mM EDTA, 100 mM NaCl, 1% SDS and 0.5 mg/ml newly added proteinase K). The lysates were incubated at 55°C with shaking for at least 3 hours or overnight. After a brief vortex, 200 µl 5 M NaCl was added and mixed on a micro-tube shaker for 5 min at RT. Then the lysates were pelleted by centrifugation at 14,000 rpm for 10 min. The supernatants containing genomic DNA were carefully removed to fresh micro-tubes and mixed with 2/3 volume isopropanol. The genomic DNA should be visible and was picked by the yellow tips to the fresh tubes with 70% ethanol. Following at least 1 hour incubation at RT, the DNA pellets were spun down (14,000 rpm, 1 min) and air-dried for 5 min. Finally pellets were dissolved in H_2O and stored at -20°C.

2.2.12 Isolation of poly(A)+ RNA from mammalian cells

50-80% confluent cells in a 15 cm petri dish (Greiner, Frickenhausen, Germany) were used for preparing poly(A)+ RNA. Medium was removed and cells were washed twice with ice-cold PBS. Then cells were immediately lysed

in 10 ml STE-SDS buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA and 0.8% SDS) containing 0.3 mg/ml proteinase K. After homogenizing, the lysate was incubated at 37°C for 30 min and the final concentration of NaCl was adjusted to 0.5 M by adding 1 ml 5 M NaCl. 1 ml (about 100 mg) Oligo-dT cellulose (type VII, Phamacia, Uppsala, Sweden) swollen in HSB buffer (10 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA and 0.1% SDS) was added and the resulting mixture was rotated at RT for at least 4 hours or overnight to allow binding of poly(A)+ RNA to the cellulose. After washing the cellulose with 10 ml HSB buffer for three times, poly(A)+ RNA was eluted with 5 ml sterile H₂O and then precipitated with 2.5 volumes pure ethanol at –20°C overnight. Samples were collected by centrifugation at 10,000 g for 1 hr (4°C) and were washed with 70% ethanol. The air-dried RNA pellets were finally dissolved in sterile H₂O and stored at –80°C.

2.2.13 Polymerase chain reaction (PCR)

All PCR reactions were performed in a total volume of 20 µl in the presence of 250 µM dNTPs, 1-2 pmol of primers, 0.25 U of Taq polymerase, 1× supplier's buffer and 2 mM MgCl₂. The reactions were carried out in a PCR thermocycler (Perkin Elmer, Norwalk, USA), using the following cycling parameters: 94°C, 1 min, 1 cycle; 94°C, 30 sec, 55°C, 30 sec and 72°C, 30 sec for a total of 30 cycles. Analysis of the PCR products were performed by a gel electrophoresis.

2.2.14 Cell culture

2.2.14.1 Mammalian cells other than ES cells

All cells were maintained at 37°C in an incubator (Forma Scientific, labortect GmbH, Göttingen, Germany) with 5% CO₂ and 95% humidity. All cells were grown in petri dishes, plates or flasks (Greiner, Frickenhausen, Germany) depending on the application. For F9 cells plastic was pre-coated with 0.1% gelatin for 5 min at RT. In general, the cells were allowed to grow to a

confluency of 80-90% and subsequently split by trypsinization and re-seeded at a lower density. Trypsin treatment was performed with removing the medium from the cells, washing cells once with PBS (Ca/Mg free) and adding 0.25% trypsin (0.05% for F9 cells). After 5-10 min incubation at 37°C, detached cells were re-suspended in fresh medium and re-seeded at the desired density. To prepare cells for storage, logarithmically growing cells were trypsinized, harvested by addition of medium, and centrifuged at 1,200 rpm for 2 min. The medium was then removed and the cells were re-suspended in 50% culture medium, 40% FCS and 10% DMSO (Fluka Chemie AG, Buchs, Switzerland) and placed in cryo-vials. After incubation on ice for 20 min, cells were transferred to -80°C for overnight before finally being stored in liquid nitrogen. To re-propagate cells, the cryo-vials were removed from liquid nitrogen and thawed at 37°C directly. The cells were then mixed with 10 ml of fresh medium, centrifuged at 1,200 rpm (to remove DMSO), and seeded on the petri dishes with fresh culture medium.

2.2.14.2 ES cells

a) Preparation of feeder cells (embryonic fibroblasts) for ES-cell culture

E14.5 pregnant mouse (CD44+/-) was sacrificed and its abdomen was swabbed by 70% ethanol. After opening up abdomen, uterus was removed and placed in a petri dish with PBS. Then the embryos were removed from uterus and extra-embryonic membranes as well as intestine, liver, heart, lung and head were cut out. The carcasses were washed in PBS again and transferred to a new dish with 0.25% trypsin solution. After mincing with scalpel blades, the mushy tissues were incubated in a total volume of 15 ml trypsin solution at 37°C for 30 min. To obtain single feeder cells, a cell strainer (Becton Dickinson, Franklin Lakes, USA) was used to filter the minced tissues. The cells were then re-suspended in the medium and plated onto 15 cm dishes (about one embryo per dish). The feeder cells can be split for propagation or frozen down at this stage. Mitotically inactivated cells prepared by gamma ray irradiation served as feeder cells for ES cell culture.

b) ES cell culture

ES cells were cultured on the top of confluent feeder cell layer on 0.2% gelatin (G-1890, Sigma) coated dishes (Becton Dickinson, Franklin Lakes, USA). The incubator (Labotect, Göttingen, Germany) used for ES cell culture was with the conditions of 8% CO₂ and 90% humidity. Thorough trypsinization (to generate single cell suspension) and a daily medium feeding interval were performed to avoid the differentiation of ES cells. A centrifugation speed of 1,000 rpm was used for spin-down of ES cells. The freezing medium for ES cells was 80% FCS and 20% DMSO. Other conditions for ES-cell culture and storage were basically the same as those for normal mammalian cells shown above.

c) Preparation of ES cells for Isolation of poly(A)+ RNA and cell extracts

ES cells on the feeder cell layer were trypsinized and re-plated on a 10 cm dish with fresh ES medium. After incubation at 37°C for 20 min to allow feeder cells to attach on the bottom of the dish, the suspending ES cells were harvested carefully. The cells were subsequently spun down (1,000 rpm, 3 min) and used for cell extract preparation or isolation of poly(A)+ RNA.

2.2.15 Stable and transient transfection of mammalian cells

2.2.15.1 Electroporation of ES cells

ES cells were split one day before transfection to allow cells in a logarithmic growth phase. Cells (about 5×10^6) were trypsinized, washed with PBS and E-buffer (5 mM Hepes in Ca/Mg free PBS, pH 7.05), and re-suspended in 0.8 ml E-buffer. After transferring to a cuvette, cells were mixed with 20 µg of linearized targeting vector. Electroporation was performed with a pulse of 400 V and a capacitance of 250 µF in a Bio-Rad gene pulser (Bio-Rad, Hercules, USA). Typically, a time const. was between 3.5-6 ms. The cuvette was

allowed to stand on ice for 10 min before seeding cells on the 10 cm dishes. 24 hours after electroporation, the selection was started by addition of G418-containing ES medium (300 µg/ml) and maintained for 7-10 days.

2.2.15.2 Lipofection

Lipofectamine, Lipofectamine Plus, Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) and Fugene 6 (Roche, Mannheim, Germany) were used for transfection of DNA plasmid, according to the instructions of the manufacturers. Appropriate antibiotics were used for selecting transfected cells depending on the selective markers of the plasmids.

Oligofectamine (Invitrogen, Karlsruhe, Germany) was used for the delivery of siRNA basically following the instruction of manufacturer. Briefly, 3×10^4 HT-29 cells were seeded on each well of a 24-well plate, which allows a cell confluency about 30% on the next day for transfection. For each well, 3 µl of 20 µM siRNA and 2.5 µl of Oligofectamine reagent were diluted in 40 µl and 7.5 µl Opti-MEM I (Invitrogen, Karlsruhe, Germany), respectively. After incubation for 7-10 min at RT, the solutions were mixed by inversion and allowed to stand at RT for a further 25 min before addition to the cells covered with 200 µl of FCS free DMEM. Four hours later 125 µl of medium containing 30% FCS was added to bring back the normal level (10%) of FCS for cell culture. Transfections were performed in 24-hr intervals to maintain the concentration of siRNA.

2.2.16 Cell extracts preparation

2.2.16.1 Whole cell extracts for enzymatic assays and immunoprecipitations

Cells were harvested by scraping with a rubber policeman, or 0.05% trypsin treatment for 5 min. Cells were washed with Ca/Mg free PBS and then counted with a counter chamber slide if necessary. After re-suspending in

NETN buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.3% NP40, 10% v/v glycerol) with fresh added protease inhibitor cocktail (Roche, Mannheim, Germany), cells were lysed on ice for 30 min, sonicated and pelleted by centrifugation (14,000 rpm, 10 min, 4°C) to collect the supernatant containing whole cell extract.

2.2.16.2 Whole cell extracts for Western blot analysis

Cells were either lysed directly in the SDS-PAGE sample buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 100 mM 2-mercaptoethanol, 20 % v/v glycerol and 0.01% bromophenol blue) before a sonication to break down the chromosomal DNA, or lysed in NETN buffer as mentioned above and the resulting cell extract was mixed with equal volume of 2× sample buffer. For both preparations, samples were boiled for 5 min before loading in a SDS-PAGE gel.

2.2.16.3 Nuclear extracts

Cells were firstly lysed in hypotonic buffer (25 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.05% NP40 and fresh added protease inhibitor cocktail (Roche, Mannheim, Germany) on ice for 5 min. Then the nuclei were pelleted by centrifugation at 200 g for 5 min (4°C) and washed once in hypotonic buffer and pelleted again. After re-suspending nuclei in a nuclear extract buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% v/v glycerol and protease inhibitor cocktail (Roche, Mannheim, Germany)), the samples were incubated on ice for additional 30 min. Finally the lysates were centrifuged at 14,000 rpm for 10 min (4°C) and the supernatants were served as nuclear extracts.

2.2.17 Determination of protein concentration

Protein concentration was determined by a method of Bradford (1976) using BSA as the standard.

2.2.18 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoretically separated on the basis of size using the method of Laemmli (1970). The resolving gels containing between 6-15% acrylamide depending on the experiment and 5% stacking gel were cast according to Sambrook *et al.* (1989). Samples were run into the stacking gel at 80-100 V and then run at 100-120 V in the separating gel with a Mini-gel system (Hoefer, San Francisco, USA).

2.2.19 Coomassie staining

SDS-PAGE gels were immersed with Coomassie stain solution (0.25% Coomassie brilliant blue R-250, 50% methanol and 10% acetic acid) for 1 hour at RT with shaking. To de-stain, the gels were incubated firstly in 50% methanol, 10% acetic acid for 1 hour, and then in 10% methanol, 10% acetic acid for several hours and several changes of the de-stain solution until the signal was clear enough.

2.2.20 Radioactive labeling of DNA probes

Radioactive labeling of DNA probes was performed with a Prime-it II Kit (Stratagene, La Jolla, USA) and purified with Nick columns (Amersham Pharmacia, Uppsala, Sweden) according to instructions of manufacturers.

2.2.21 Southern blot analysis

The DNA samples were electrophoretically separated in the agarose gel containing ethidium bromide and photographed with a ruler under UV light. The gels were then incubated in 0.25 M HCl for 10 min, denatured in 0.5 M NaOH/1.5 M NaCl for 30 min, neutralized in 10x SSC buffer (1.5 M NaCl, 0.15 M sodium citrate dihydrate) for 15-45 min until the pH value below was 9. Then DNA was transferred to GeneScreen Plus membranes (NEN life science

products, Inc., Boston, USA) with 10× SSC buffer using a capillary blotting method. After the overnight transfer, the membranes were soaked with 0.4 M NaOH for 1 min, 0.2 M Tris-HCl pH 7.5, 1× SSC for 1 min and 1× SSC for 2 min. UV light crosslinking was then performed for the air-dried membranes to immobilize the DNA. The membranes were pre-hybridized in the glass tubes for 2 hours at 65°C in 10 ml Church buffer (1 M NaPO₄ pH7.2, 1 mM EDTA and 7% SDS) and then hybridization was carried out by addition of 100 µl radioactively labeled DNA probe (about 10⁷ cpm, denatured at 95°C for 5 min). After 12 hours, the membranes were washed two times for 20 min each at 65°C in 2× SSC and 0.5× SSC containing 0.1% SDS. The membranes were finally sealed in the plastic bags and exposed to Hyperfilm MP (Amersham Pharmacia, Buckinghamshire, UK) at –80°C for several hours to days.

2.2.22 Northern blot analysis

The method is based on the protocol of Lehrach *et al.* (1977). Briefly, poly(A)+ RNA was separated in a 1% formaldehyde MOPS gel and then the gel was soaked in sterile H₂O for 5 min to remove the formaldehyde. After rinsed in 10× SSC, RNA was transferred to the membranes and hybridized with appropriate DNA probe as mentioned above. For the human samples using murine DNA probes, the temperature for hybridization and washing was reduced to 58°C. Signal was detected using MP Hyperfilm as above and a phosphoimager (FLA-3000, Fuji) and the software Aida 2.11 (Raytest, Straubenhardt, Germany) were used for quantification of the density of the bands.

2.2.23 Stripping DNA probes

To allow re-use of the Southern or Northern blots, radioactively labeled DNA probe was stripped by incubating the membranes in a strip buffer (0.1× SSC, 1% SDS) at 95°C for 20 min. The process can be repeated in case a strong

radioactivity remained. The membranes were then used for re-probing from the pre-hybridization step as usual.

2.2.24 Western blot analysis

Proteins in SDS-PAGE gels were transferred onto methanol pre-soaked Immobilon-P membranes (Millipore, Bedford, USA) in a transfer chamber (Bio-Rad, Hercules, USA) containing blotting buffer (20 mM Tris, 192 mM glycine and 10% v/v methanol) at 30 V overnight (4°C). A semi-dry blotter (H.Hölzel, Wörth/Hörlkofen, Germany) was also used for blotting using the same transfer buffer at a current of 1 mA/cm² for 1 hour. After the transfer was complete, membranes were incubated in TBST buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl, 0.05% Tween 20) supplemented with 5% non-fat milk powder at RT for 1-2 hours to reduce unspecific binding. Primary antibodies were diluted in the blocking TBST buffer at a concentration recommended by the supplier (generally 1:500-1:1000). Then the membranes were incubated in the primary antibody-containing buffer for a further 1-2 hours at RT or 12 hours at 4°C. The membranes were then washed three times with TBST buffer for 5 min each. An appropriate secondary HRP-conjugated antibody was added and incubated for additional 1 hour at RT. Once the membranes were washed again with TBST buffer for three times, detection of specific protein signals were achieved by enhanced chemiluminescence using ECL Western blotting detection reagents and ECL Hyperfilm (Amersham Pharmacia, Buckinghamshire, UK) following the manufacturer's instructions. The Aida 2.11 program was used for quantification of the density of the signals.

2.2.25 Stripping Western blot membrane

To utilize Western blots more than a single use, the membranes were stripped by the following method. They were incubated in a strip solution (62.5 mM Tris-HCl pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol) at 50°C for 40 min with shaking. The membranes were then washed twice with TBST for 5 min

each time, blocked with 5% milk buffer as usual and used for Western blot probing again.

2.2.26 Immunoprecipitation

The cell extracts in NETN buffer were used for immunoprecipitation. 800 μ l of the cell extract (from about 2×10^7 cells generally) was mixed with 3-5 μ g of an appropriate primary antibody and incubated at 4°C for 2 hours on a tube-rotator. Then 50-100 μ l (about 3-6 mg) of protein A-Sepharose (CL-4B, Amersham Pharmacia, Uppsala, Sweden) in NETN buffer was added and incubated with rotation at 4°C overnight. The beads were then precipitated by centrifugation at 2,500 rpm for 5 min and washed 3-4 times with NETN buffer. Finally the immunoprecipitates were re-suspended in NETN buffer for enzymatic assays or re-suspended in 2 \times SDS-PAGE sample buffer and boiled for 5 min for Western blot analysis.

2.3 Analytical Methods

2.3.1 Pulse-labeling and pulse-chase experiment

Cells (pre-treated with or without VPA for 24 hrs) of 70-90% confluency grown on 10 cm dishes were washed twice with labeling medium (Met/Cys/L-Glu deficient DMEM (Sigma) supplemented with 2 mM L-glutamine). After starving the cells in 5 ml labeling medium (with or without VPA) for 30 min (37°C), cells were metabolically labeled with 3 ml labeling medium (with or without VPA) containing 0.1 mCi/ml Pro-mix [35 S] (Amersham Pharmacia, Buckinghamshire, UK) for 1 hour (for pulse-labeling experiments) or 3 hours (for pulse-chase experiments) in a cell culture incubator. Then cells that served as pulse-labeling samples or “chase time 0 hour” samples were washed with ice-cold PBS for three times and lysed in 1 ml NETN buffer with protease inhibitor cocktail and 30 μ M MG-132. Cells for pulse-chase experiments were washed with normal cell culture medium (with or without VPA) and incubated in 5 ml normal medium for additional 3 hours or 6 hours

to chase newly synthesized radioactive proteins. Cell extracts were prepared as above and all extracts were in turn used for immunoprecipitating specific proteins. Aliquots of cell extracts before and after immunoprecipitation and aliquots of immunoprecipitates were saved for controls. The immunoprecipitates were subjected to run in the SDS-PAGE gel and the gel was then dried with a vacuum gel-dryer (Bio-Rad, Hercules, USA) at 80°C for 2 hours. The signal was detected by exposing the gel to MP Hyperfilm at -80°C for several days. Quantification of the signals was carried out by using the Aida 2.11 program.

2.3.2 *In vivo* ubiquitination assay

Confluent HEK293T cells were split the day before transfection at a ratio of 1:6 in 10 cm dishes. Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) was used to transfect cells with 10 µg of pHis₆-Ubi, following the instruction of the manufacturer. For VPA-treated samples, VPA was added 12 hours after transfection and maintained for a further 36 hours. MG-132 (Calbiochem, La Jolla, USA), a potent proteasome inhibitor, was added (f.c. 30 µM) into the medium 4 hours prior to harvest to enhance accumulation of the poly-ubiquitinated proteins. The cells were then washed twice with ice-cold PBS and 1/10 amount was lysed in SDS-PAGE sample buffer to determine the total amount of the protein of interest by Western blotting. Remaining cells were re-suspended in 7 ml Guanidine lysis buffer (100 mM Na₂HPO₄/NaH₂PO₄ pH 8, 10 mM Tris-HCl pH 8, 6 M guanidine-HCl, 10 mM 2-mecaptoethanol and 5 mM imidazole fresh added), and 75 µl of Ni²⁺-NTA-Agarose beads (Qiagen, Hilden, Germany) was added to allow binding of the His-ubiquitinated proteins. After incubation at RT overnight on a rotating wheel, beads were precipitated by centrifugation at 2,000 rpm for 5 min. Then the beads were consecutively incubated for 10 min each in 0.75 ml Guanidine lysis buffer, Urea buffer (100 mM Na₂HPO₄/NaH₂PO₄ pH 8, 10 mM Tris-HCl pH 8, 8 M urea, 10 mM 2-mecaptoethanol fresh added), buffer A (100 mM Na₂HPO₄/NaH₂PO₄ pH 6.3, 10 mM Tris-HCl pH 6.3, 8 M urea, 10 mM 2-mecaptoethanol fresh added), buffer A with 0.2% Triton X-100, and buffer A

with 0.1% Triton X-100. Finally His-ubiquitinated proteins were eluted from the beads by incubating in 75 μ l elution buffer (150 mM Tris-HCl pH 6.8, 200 mM imidazole, 5% SDS, 30% v/v glycerol, 0.72 M 2-mercaptoethanol) for 1 hour at RT. Supernatants containing ubiquitinated proteins were then subjected to Western blot analysis as usual.

2.3.3 Hoechst DNA staining

Logarithmically growing cells were harvested by trypsinization and washed once in Ca/Mg free PBS. Cells were then re-suspended in 200 μ l PBS and an equal volume of fixation solution (8% formaldehyde, 2% methanol in PBS) was added. After 10 min incubation at RT, cells were permeabilized by addition of 30 μ l 10% NP40 and incubated for another 10 min. Finally cells were stained by addition of 400 μ l PBS/2% FCS containing 2 μ g/ml Hoechst dye (DNA bisbenzimidazole dye H33258). Samples were used for cell cycle analysis by FACSing or detecting apoptotic cells under microscope.

2.3.4 Flow cytometry (FACS)

2.3.4.1 Cell cycle analysis

Cells stained with Hoechst dye were analyzed by flow cytometry (FACS Star Plus, Becton Dickinson, Franklin Lakes, USA) having a UV-light filter (325 nm) for measuring Hoechst stained DNA contents. Data analysis was performed using the supplied CellQuest pro software.

2.3.4.2 Detection of GFP-positive cells

The pCMV-mRPD3-2.1 (murine HDAC2 expression vector) or the empty control vector pcDNA3.1(+) was co-transfected with the GFP expression vector pCMV-GFP (at a ratio of 5:1) into HT-29-APC cells using Lipofectamine 2000 reagent. 12 hours later, the transfected cells were trypsinized and re-plated to normalize the variation in transfection efficiency, and ZnCl₂ (f.c. 100

μM) was added in ZnCl_2 -treated samples. The attached cells were harvested at various time points (24-72 hrs) after ZnCl_2 -treatment and used for detection and analysis of GFP-positive cells at 488 nm (extinction) and 530 ± 15 nm (emission) by flow cytometry.

2.3.5 Apoptosis assay

2.3.5.1 Microscopic analysis (DNA fragmentation-based apoptosis assay)

Cells stained with Hoechst dye were counted under a microscope (Nikon, Japan) with the UV-light filter. Cells possess condensed or fragmented nuclei were counted as apoptotic cells. Totally 200 cells from 5-10 fields were counted for each sample, and the cells were photographed by a digital camera (Hamamatsu, Japan) and supplied OpenLab 2.2.5 software.

2.3.5.2 Using Cell Death Detection ELISA^{PLUS} Kit

The Cell Death Detection ELISA^{PLUS} Kit (Roche, Mannheim, Germany) is a photometric enzyme-immunoassay kit for *in vitro* qualitative and quantitative determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) produced after programmed cell death (apoptosis). The detection was performed following the manufacturer's recommendations using 3×10^4 - 10^5 cells for each assay.

2.3.6 Immunohistochemistry (IHC)

The tissue array slides (BA2, Biocat, Heidelberg, Germany) containing various human normal organs and tumors were used for immunohistochemistry. The formalin-fixed, paraffin-embedding slides were dried at 58°C for 1 hour, then deparaffined in xylene twice for 10 min each. Following re-hydrated in 100%, 95% and 75% ethanol twice for 3 min each, slides were immersed in tap water for 5 min. Endogenous peroxidase was quenched by incubating slides

in 3% hydrogen peroxide solution for 10 min. After slides were incubated with blocking serum for 30 min, an anti-HDAC2 antibody (Zymed, rabbit polyclonal, 1:50 dilution) or an anti- β -catenin antibody (Santa Cruz, goat polyclonal, 1:50 dilution) was used to probe the specific protein. Following incubation for 90 min at RT, slides were washed in PBS for three times. Then slides were incubated with biotin-conjugated anti rabbit (or goat) IgG antibody (1:100 dilution) for 30 min. After washing again in PBS, slides were incubated with avidin-biotin complex (Vector, Burlingame, USA) for another 30 min. Slides were stained with NovaRED substrate kit (Vector, Burlingame, USA) following the manufacturer's instruction. The reaction was stopped by washing slides in tap water. Slides were then counterstained in hematoxylin for 10 sec and briefly rinsed with PBS. After dehydrating in 70%, 95%, 100% ethanol twice for 2 min each and cleaning in xylene twice for 2 min each, slides are finally mounted with CVMount solution (Leica, Nussloch, Germany) and subjected to observation under microscope. Slides were photographed by a digital camera and supplied software ProgRes 5.0 (Jenoptik, Germany).

2.3.7 *In vitro* histone deacetylase activity assay (HDAC assay)

HDAC activity was measured either in cell extracts or immunoprecipitates with antibodies against distinct HDACs or corepressors. Reactions were started by addition of 2 μ l (about 20,000 cpm) of 3 H-labeled hyperacetylated histone substrates into a mixture of 50 μ l of enzymatic source and assay buffer (25 mM Tris-HCl pH 7.4, 75 mM NaCl, 0.2 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF) in a total volume of 200 μ l. The HDAC inhibitors TSA and VPA were added 15 min prior to addition of substrate and the reactions were continued for 90 min at 37°C. The reactions were stopped with adding 21 μ l 10 \times stop solution (1.2 M HAc/7.2 M HCl) and the released 3 H-acetate was extracted with 400 μ l ethyl acetate. After a vigorous vortex and a brief centrifugation, 300 μ l of organic phase (supernatant) was removed to vials with 3 ml liquid scintillation cocktail solution (Packard, Groningen, the Netherlands) and the radioactivity that represents HDAC activity was measured with a liquid scintillation counter (Kendro, Asheville, USA).

2.3.8 *In vitro* histone acetyltransferase activity assay (HAT assay)

Nuclear extracts were incubated with ^3H -labeled acetyl-CoA (Amersham Pharmacia, Buckinghamshire, UK) in the assay buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany) and TSA (300 nM) at 37°C for 60 min. Then the reaction mixtures were separated by 15% SDS-PAGE. The gels were subsequently stained with Coomassie blue for visualizing histones, incubated in the Amplify solution (Amersham Pharmacia, Buckinghamshire, UK) for 30 min, dried under vacuum at 80°C for 2 hours and exposed to Hyperfilm-MP films.

2.3.9 *In vitro* alkaline phosphatase activity assay (ALP assay)

The method was modified from the protocol of Reese *et al.* (1981). Cells were lysed in NETN buffer and were frozen, thawed and vigorously vortexed prior to protein concentration and alkaline phosphatase determinations. The ALP activity was measured by incubating 50 μl of the samples in 150 μl assay buffer (0.64 M 2-amino-2-methyl-1-propanol pH 10.3, and 1 mM MgCl_2) containing 20 mM p-nitrophenylphosphate (p-NPP) substrate at 37°C for 30 min to 6 hours. The amount of p-nitrophenol liberated was determined with a spectrophotometer (Bio-tek, Winooski, USA) at 415 nm to represent the ALP activity.

3. Results

3.1 Evidence that HDAC2 is regulated by PPAR δ

F9 teratocarcinoma cells have properties similar to those of embryonic cells and can be induced to differentiate upon appropriate stimuli, e.g. retinoids and cAMP signaling. Therefore, F9 cells have been used as a simple model for the study of the mouse embryo development (Lehtonen *et al.*, 1989). Our laboratory previously showed that VPA and its teratogenic derivatives selectively activate PPAR δ , and induce a specific type of F9 cell differentiation (Lampen *et al.*, 1999; Werling *et al.*, 2001). To understand the roles of PPAR δ for F9 cell differentiation, our laboratory generated sub-clones of F9 cells in which PPAR δ antisense RNA is constitutively expressed and PPAR δ expression is knocked down consequently. Due to the absence of appropriate antibodies against PPAR δ suitable for Western blot analysis, the deficiency of this receptor was analyzed indirectly by a “band-shift” assay. PPAR δ -deficient clones lost a specific protein-DNA complex, and antibodies against PPAR δ caused a “super-shift” of the complex in wild-type F9 cells but not in the deficient sub-clones. These PPAR δ -deficient clones showed a partially differentiated phenotype including reduced proliferation rate and lack of response to VPA, e.g. resistant to the induction of AP-2 protein (Werling *et al.*, 2001). Since VPA was identified as a histone deacetylase (HDAC) inhibitor recently (Göttlicher *et al.*, 2001; refer to section 3.3), the status of histone acetylation upon VPA-treatment was evaluated in F9 cells. Interestingly, VPA could not induce the accumulation of histone acetylation in PPAR δ -deficient clones, unlike in the parental F9 cells and other cell lines (Göttlicher, unpublished data). Based on the knowledge that histone acetylation is controlled by two groups of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), loss of histone hyperacetylation upon VPA-treatment in PPAR δ -deficient clones suggested that PPAR δ might play a role in regulation of HATs and/or HDACs. The proposition was tested in F9 cells and mouse ES cells.

3.1.1 Reduction of HDAC activity in PPAR δ -deficient F9 cells

Bulk HDAC activity in PPAR δ antisense RNA expressing F9 clones was investigated by an *in vitro* HDAC assay (refer to section 2.3.7). All three clones showed a decrease by about 30% compared to parental F9 cells with respect to HDAC activity in nuclear extracts (Fig. 3.1). Based on the fact that class I HDACs are predominantly localized in nucleus whereas class II enzymes are actively maintained in the cytoplasm, this observation was a hint that one or several of the class I enzymes maybe reduced as a consequence of PPAR δ -deficiency. HAT activity was also tested (refer to section 2.3.8). Although nuclear extracts from PPAR δ -deficient clones showed higher HAT activity than those from parental F9 cells, there was no consistency among those clones (Fig. 3.2). I focused on HDACs for the further analysis.

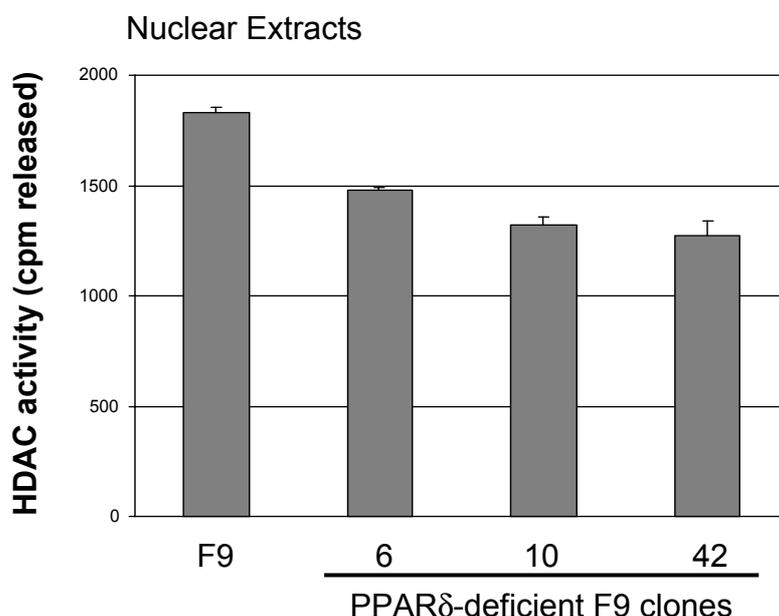


Figure 3.1 HDAC activity in the F9 cell line and its PPAR δ -deficient clones. HDAC activities in nuclear extracts from F9 cells and three PPAR δ -deficient clones 6, 10 and 42 were determined by *in vitro* HDAC assays. Equal protein amount (100 μ g) from the nuclear extract of each cell line was used. Data are means \pm SD from three measurements corrected for non-enzymatic release (cpm \leq 50).

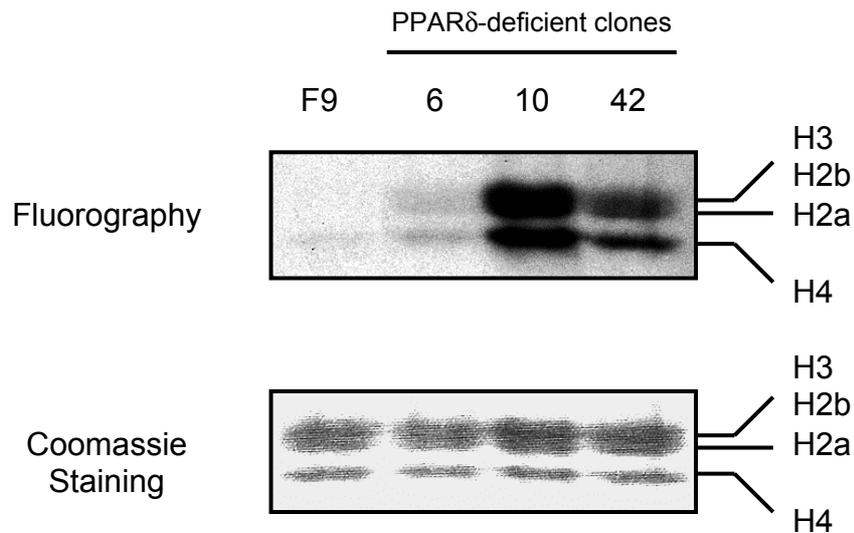


Figure 3.2 HAT activity in the F9 cell line and its PPAR δ -deficient clones. HAT activities of F9 cells and PPAR δ -deficient clones were determined by an *in vitro* HAT assay. Nuclear extracts were used as the enzymatic source and endogenous histones in nuclear extracts served as substrate. Fluorography (upper panel) of the ^3H -acetyl-histone shows the incorporation rates of acetate from ^3H -acetyl-CoA into histones, which represent the HAT activities. The Coomassie staining of core histones (bottom panel) shows that approximately equal amounts of nuclear extracts were used for the assay. Similar results were obtained in 2 additional independent experiments.

3.1.2 Selective reduction of HDAC2 expression in PPAR δ -deficient F9 cells

The reduced HDAC activity in PPAR δ -deficient F9 sub-clones could be due to either lower HDAC expression levels or decreased specific HDAC activity. Since PPAR δ is a transcription factor, the expression of HDACs and corepressors that might influence histone acetylation and HDAC activity was investigated in various F9 clones by Western blot analysis. I evaluated class I enzymes (HDAC1, 2 and 3), the class II enzyme HDAC5 and the corepressor mSin3B. Only HDAC2 was found to be severely reduced to a non-detectable level in PPAR δ -deficient (antisense RNA expressing) clones by Western blotting. HDAC1 appeared to be down-regulated slightly while other HDACs and mSin3B were not changed in these cell lines (Fig. 3.3 A). Further Northern blot analysis clearly showed that HDAC2 mRNA was reduced to virtually non-detectable levels which most likely explains the loss of HDAC2 protein (Fig. 3.3 B).

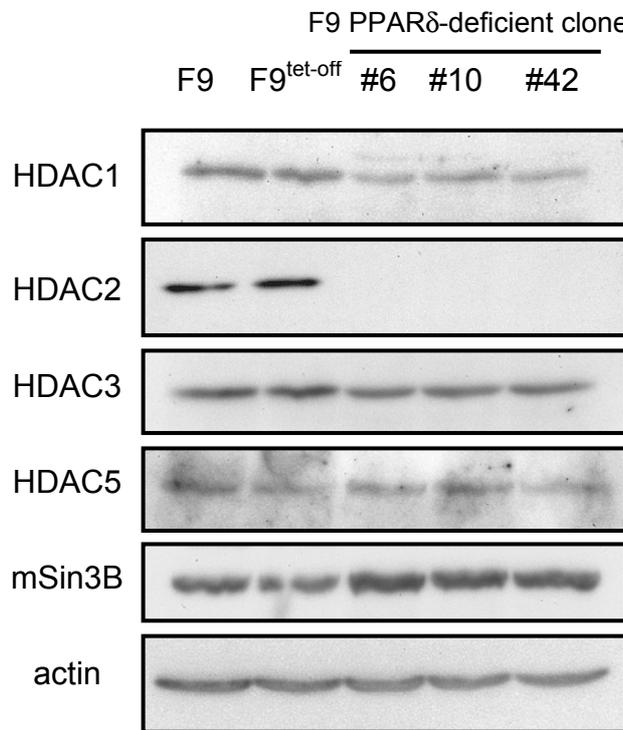
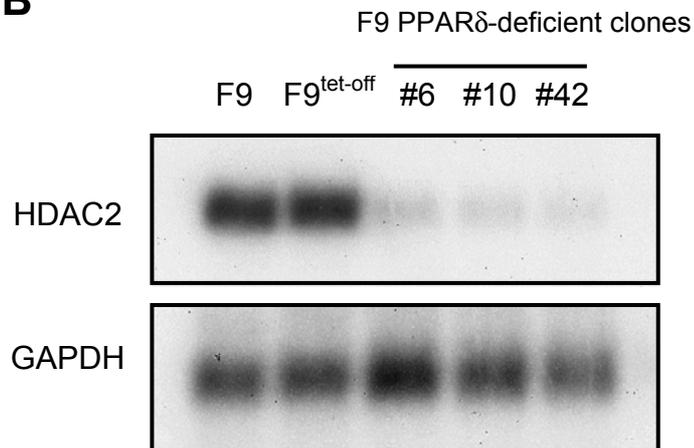
A**B**

Figure 3.3 Expression of HDACs and corepressor in the F9 cell line and its PPAR δ -deficient clones. (A) The protein levels of HDACs and corepressor mSin3B were detected by Western blot analysis. Equal loading was confirmed by re-probing the blot with anti-actin antibody. (B) The mRNA levels of HDAC2 were detected by Northern blot analysis. The same blot was re-probed with GAPDH to serve as the loading control. F9^{tet-off} is the parental cell line of PPAR δ -deficient (PPAR δ antisense RNA expressing) clones, although antisense RNA expression is constitutive rather than controlled by tetracycline.

3.1.3 Selective down-regulation of HDAC2 in PPAR δ ^{+/-} ES cells

A genetically cleaner system to reduce PPAR δ was desired to confirm the observations in F9 cells. Therefore, mouse PPAR δ ^{+/-} ES cells were generated and subjected to HDAC expression analysis. Two clones initially generated for producing PPAR δ knockout mice contain a mutated PPAR δ allele that was disrupted by homologous recombination. The targeting strategy by which both DBD and LBD of PPAR δ are disrupted does not allow the mutated allele to generate a functional protein (Fig. 3.4 A). As shown by Southern blot analysis, wild-type (wt) ES cells produced only one 4.9-kb band from wt alleles, whereas two targeted clones showed an additional 2.3-kb band from the mutated allele (Fig. 3.4 B). Thus, the expression of wt PPAR δ could be decreased in heterozygous ES clones containing only one wt allele, which was proven by Northern blot analysis (Fig. 3.5 A). The band (about 4-kb) representing PPAR δ mRNA transcribed from the wt allele is down-regulated by about 40-60% in two PPAR δ ^{+/-} ES clones compared to the wt parental ES cells. One clone (E10) appeared slightly higher expression that might be due to the clonal variation. No additional band was detectable in PPAR δ ^{+/-} ES-cell clones, possibly because the “neo” cassette (in an inverted transcriptional orientation) interferes with the transcription of the mutated allele, or the mutated RNA products are not stable. These PPAR δ ^{+/-} clones maintained the undifferentiated morphology of wt ES cells. Therefore, the PPAR δ ^{+/-} ES cells could serve as the second and better defined model system, compared to the F9 cells, for analyzing HDAC expression upon reduction of PPAR δ expression.

As expected, both mRNA and protein levels of HDAC2 were reduced by 40-60% in PPAR δ heterozygous clones (Fig. 3.5). Interestingly, the clone (D71) that expresses slightly less PPAR δ also showed a relative decrease of HDAC2 expression, suggesting a correlation between two mRNAs. The expression of other class I HDACs, HDAC1 and 3, was investigated by Western blotting, and was not reduced in PPAR^{+/-} clones (Fig. 3.5 B). Taken

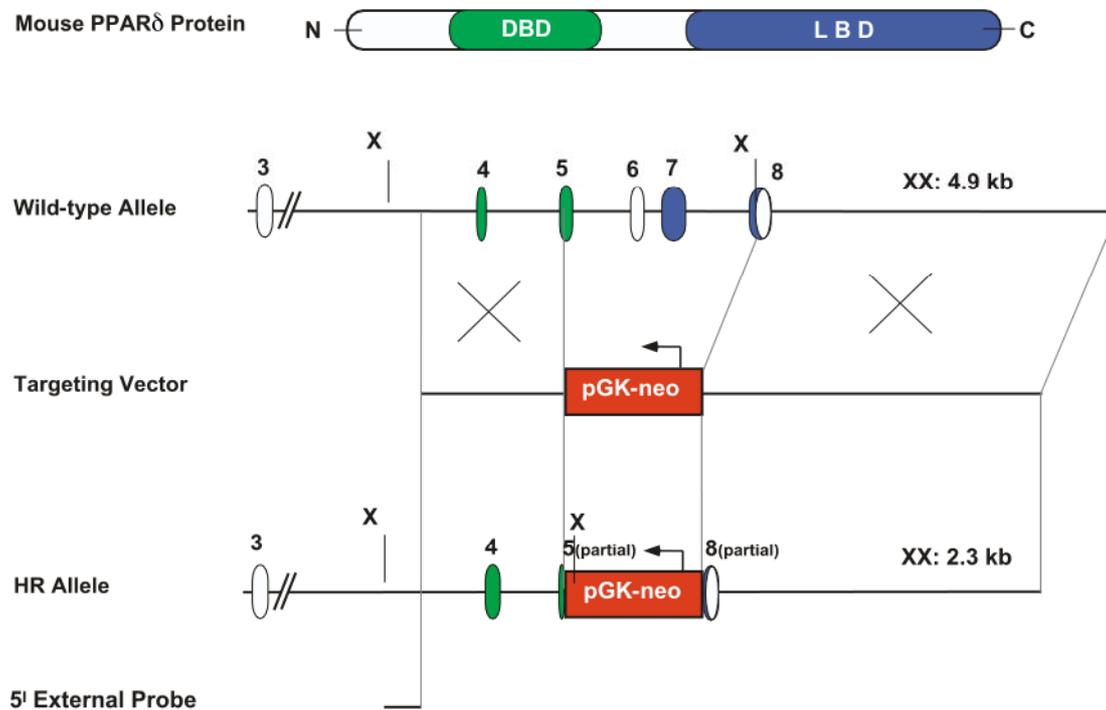
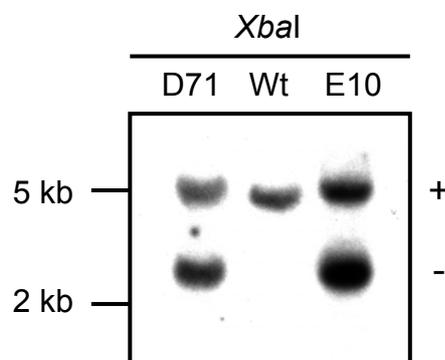
A**B**

Figure 3.4 Targeted disruption of the PPAR δ gene. (A) Schematic diagram (from top to bottom) of the mouse PPAR δ protein, the wild-type gene locus, the targeting vector, the mutated allele after homologous recombination, and the 5' external probe for Southern blot hybridization. The genomic fragment containing part of exon 5, exon 6 and exon 7 plus part of exon 8 which encode the carboxyl end of DNA binding domain (contains the second zinc finger structure), hinge region and ligand binding domain respectively (Magge and Guardiola-Diaz, 2002), was deleted and replaced by a neomycin resistant gene. X: *Xba*. (B) Southern blot analysis of genomic DNA isolated from G418-resistant ES cell clones. After digestion with *Xba*, a 4.9-kb fragment and a 2.3-kb fragment, which are recognized by the 5' external probe, were generated from the wild-type allele and the homologous recombinant allele, respectively. Two of the homologous recombinant clones, termed D71 and E10, are shown.

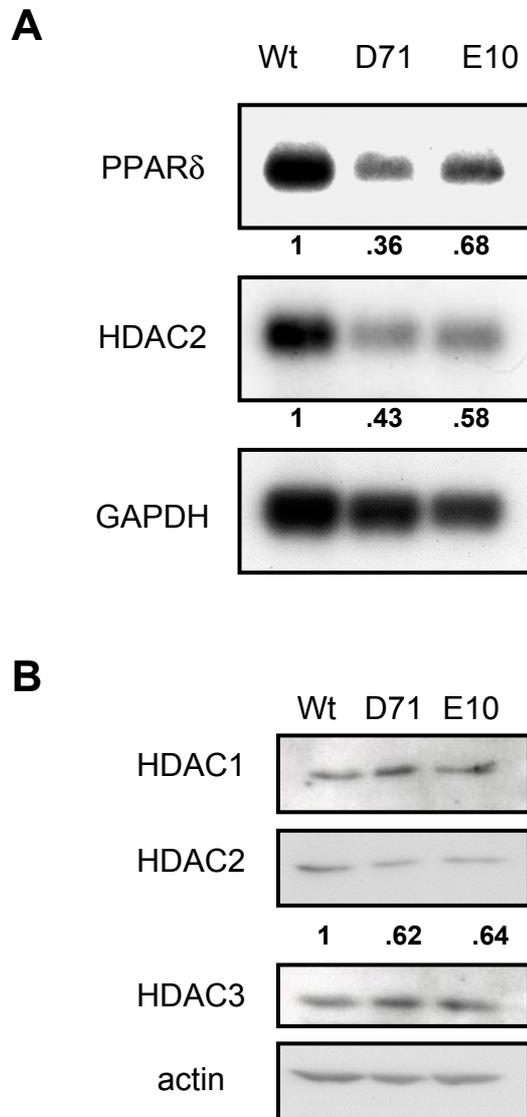


Figure 3.5 Expression of class I HDACs in wild-type and PPAR δ +/- ES cells. (A) The mRNA levels of PPAR δ and HDAC2 from parental wild-type and PPAR δ +/- ES cells (D71 and E10) were determined by Northern blot analysis. GAPDH signals served as the loading control. (B) The protein levels of HDAC1-3 were tested by Western blot analysis. The blot was stripped and re-probed with an anti-actin antibody to ensure equal loading. The numbers below the panels represent relative amounts to wt ES cells upon normalization with loading control signals. One representative out of two similar experiments is shown.

together, the analysis of mutant ES cells defective in one of the PPAR δ alleles clearly showed a selective down-regulation of HDAC2 mRNA and protein, supporting that PPAR δ contributes to the regulation of HDAC2 expression.

3.1.4 Induction of HDAC2 upon PPAR δ overexpression

Data from F9 and ES cells showed that HDAC2 was down-regulated under conditions of reduced PPAR δ expression, suggesting that PPAR δ regulates HDAC2 expression either directly or indirectly. To further prove this, a gain-of-function experiment was carried out by means of PPAR δ overexpression. In F9 cells, the induction of HDAC2 was observed upon transfection with a PPAR δ , but not GFP expression vector (Fig. 3.6). However, this effect was not seen in PPAR δ -deficient sub-clones, and the HDAC2 was still non-detectable upon PPAR δ vector transfection (Fig. 3.6). Monitoring GFP expression by FACS analysis pointed out that the transfection efficiency in PPAR δ -deficient sub-clones was very low (8-12%) compared to that in parental F9 cells (35-45%) (data not shown). This could be an explanation for the failure to observe HDAC2 induction in the F9 sub-clones. The constitutively expressed antisense RNA could also antagonize ectopic PPAR δ expression, which might be another explanation.

3.1.5 Unchanged HDAC2 expression upon ligand-activation of PPAR δ

The so far presented work indicates that the expression level of PPAR δ positively controls HDAC2 expression. PPAR δ is a ligand-activated sequence-specific NR that activates target gene expression via ligand-binding. If PPAR δ directly regulates HDAC2 expression upon binding to its promoter, activating PPAR δ with an appropriate ligand should give rise to an elevated expression of HDAC2. This assumption was tested using cPGI that is a selective ligand for PPAR δ and activates its transcriptional activity (Forman *et al.*, 1997). In F9 cells, HDAC2 expression was not enhanced upon cPGI-treatment at protein level (Fig. 3.7 A). Similarly, cPGI also did not change HDAC2 level in HT-29, a

human colorectal tumor cell line (Fig. 3.7 B). The observations could suggest that an endogenous ligand is persistent at a concentration that is sufficient for full activation of PPAR δ . Alternatively, PPAR δ might regulate HDAC2 in an agonistic ligand-independent manner. The presumption of endogenous ligand is supported by the finding that also an appropriate PPAR δ -responsive reporter gene is active in F9 cells depending on levels of PPAR δ but not the presence of additional ligand (Göttlicher, personal communication).

3.1.6 Summary of this section

This section shows that the expression of HDAC2, unlike other class I HDACs, is positively regulated by PPAR δ in mouse cell lines. Exact mechanisms underlying this process are still unclear, but could involve direct transcriptional regulation or indirect regulation via other factors.

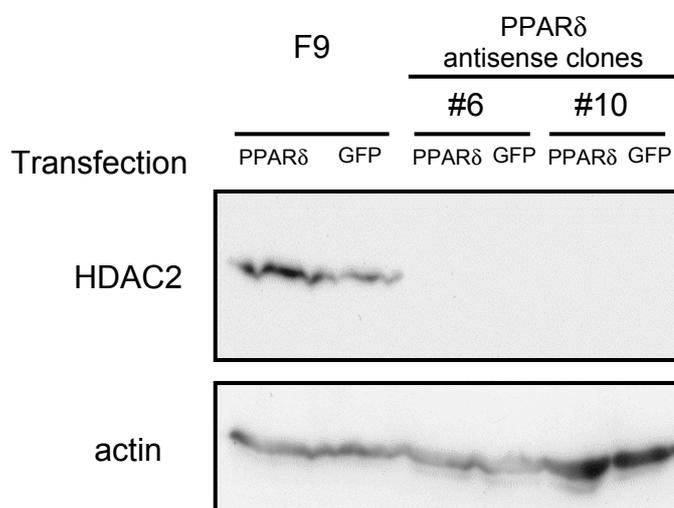


Figure 3.6 Induction of HDAC2 upon PPAR δ overexpression in F9 cells. F9 cells and PPAR δ antisense RNA expressing sub-clones (#6 and #10) were transfected with pUbi-FAAR (mouse PPAR δ expression vector) or pUbi-GFP (GFP expression vector used as a control). Whole cell extracts were prepared 48 hrs after transfection and subjected to Western blot analysis for detection of HDAC2. The same blot was re-probed with an anti-actin antibody to confirm equal loading. One representative out of two similar experiments is shown.

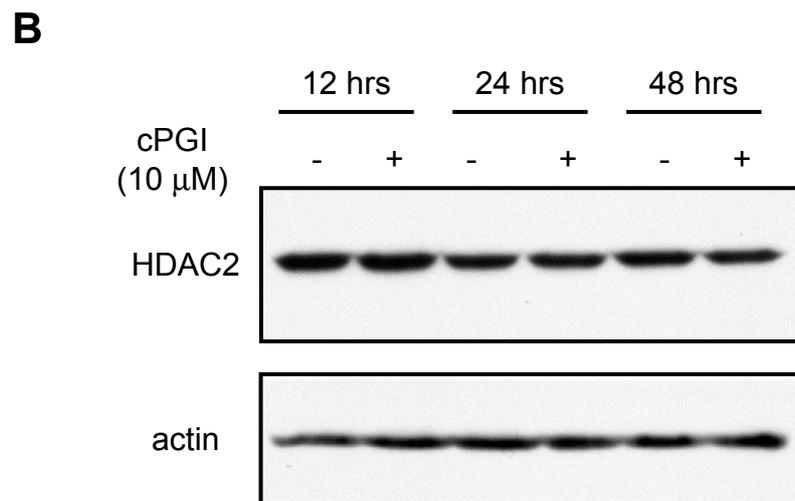
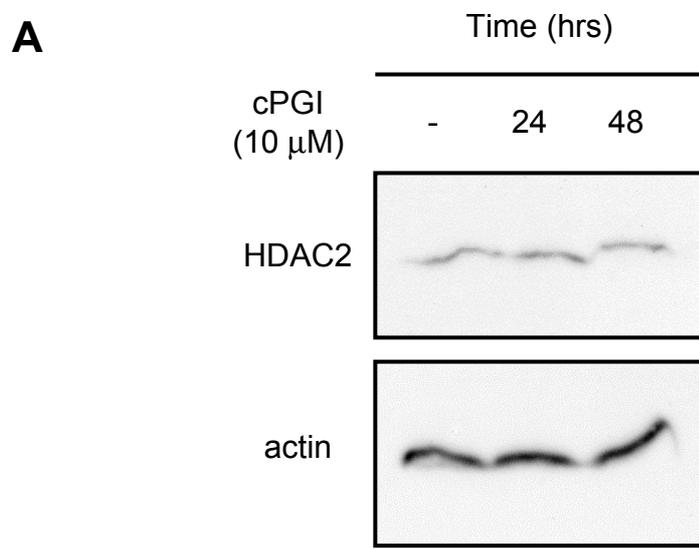


Figure 3.7 Activation of PPAR δ by cPGI does not induce HDAC2 expression. Cells were treated with 10 μ M cPGI (+) or ethanol solvent as the control (-). Whole cell extracts were prepared at several time points after treatment as indicated and subjected to Western blot analysis of HDAC2. Re-probing the same blot with an anti-actin antibody confirmed equal loading. **(A)** F9 cells. **(B)** HT-29 cells. Experiments were performed three times in F9 cells and twice in HT-29 cells with similar results.

3.2 Roles of HDAC2 in transformed phenotype of colorectal cancer cells

PPAR δ is believed to be crucially involved in colorectal tumorigenesis (He *et al.*, 1999; Gupta *et al.*, 2000; Park *et al.*, 2001), although this proposition is challenged by the observation that PPAR δ germ-line mutation only has moderate effects on colonic polyps formation in an APC^{min} mouse model with a spontaneous mutation of the APC gene (Barak *et al.*, 2002) (refer to 1.2.5.2.2). PPAR δ is a potential down-stream target gene of APC/ β -catenin signaling, which is negatively regulated by wt APC tumor suppressor and frequently over-activated in colorectal cancers. In cancer cells, inactivating APC mutations (or activating β -catenin mutations if APC is functional) lead to an elevated β -catenin/Tcf-mediated transcription. Consequently, expression of PPAR δ as well as of several other target genes, e.g. c-myc and cyclin D1, is up-regulated via β -catenin/Tcf-responsive elements in their promoters and reduced upon restoring wt APC (He *et al.*, 1999). Despite the knowledge that PPAR δ plays a critical role in colorectal tumorigenesis, the downstream targets of PPAR δ that could contribute to the process are unknown. The proposition that HDAC2 could be regulated by PPAR δ shown in the previous section suggested a potential role of aberrantly high HDAC2 expression in pathogenesis of colorectal cancer. It has been shown that aberrant recruitment of HDAC activity is crucially involved in pathogenesis of certain cancer forms originally from hematopoietic cells, such as APL and AML (Krämer *et al.*, 2001; Marks *et al.*, 2001), whereas there is no examples in non-hematopoietic tumors to date. Based on these findings together with the described reports from the literatures, the following hypotheses were proposed and tested: 1) HDAC2 is a downstream target of APC/ β -catenin signaling possibly mediated by PPAR δ . HDAC2 is up-regulated due to inactivating mutations of APC (or activating mutations of β -catenin) in colon cancer cells in which β -catenin/Tcf-mediated transcription is elevated and PPAR δ is overexpressed subsequently; 2) HDAC2 up-regulation plays a

critical role for transformed phenotype of colon cancer cells containing APC mutations (or β -catenin mutations) (Fig. 3.8).

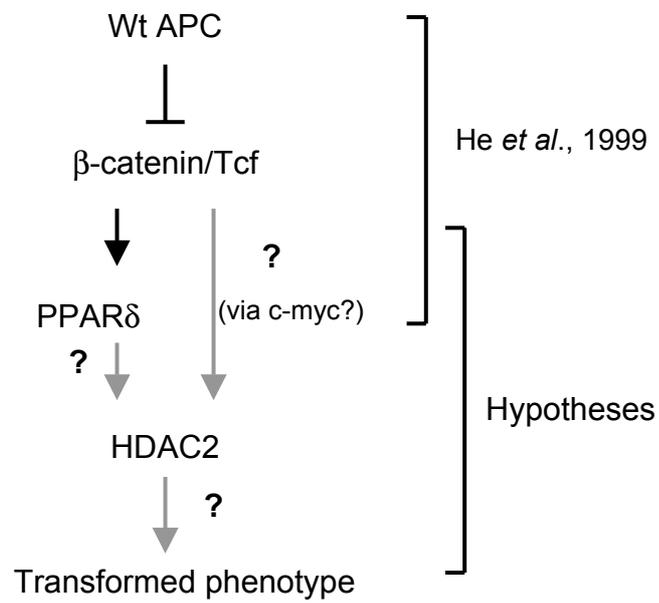


Figure 3.8 The schematic sketch of proposed and hypothetical pathways in colorectal tumorigenesis

3.2.1 Reduced HDAC2 expression upon restoring wt APC in HT-29 cells

HT-29 cells are colorectal cancer cells that contain two mutant APC alleles coding for C-terminal truncated non-functional proteins instead of intact APC. Morin *et al.* (1996) generated HT-29-APC and control HT-29- β gal sub-clones in which full-length functional APC and β -galactosidase (as a control) are expressed, respectively, under the zinc inducible part of metallothionein promoter. I firstly tested the first hypothesis that HDAC2 expression is regulated by APC by using the APC-inducible HT-29 cell line. Upon restoring wt APC expression by 100 μ M ZnCl₂-treatment (Morin *et al.*, 1996), HDAC2 as well as PPAR δ mRNA expression were significantly down-regulated, suggesting that both are under the control of APC/ β -catenin pathway (Fig. 3.9, left panels). PPAR δ has been shown to be reduced as a result of wt APC expression in HT-29 cells (He *et al.*, 1999). HDAC2 protein level was also dramatically reduced upon zinc-treatment in HT-29-APC cells (Fig. 3.10, left panels), whereas another class I HDAC, HDAC3, expression was not changed, suggesting a selective effect on HDAC2. The same zinc-treatment did not affect HDAC2 expression in the control HT-29- β gal cell line in which β -galactosidase instead of APC was inducible (Fig. 3.9 and 3.10, right panels), indicating that ZnCl₂ itself does not affect HDAC2 expression and supporting the interpretation that down-regulation of HDAC2 upon zinc-treatment is indeed due to wt APC expression. HDAC2 down-regulation occurred about 8 hours after zinc-treatment and reached the peak 40 hours later (Fig. 3.9 and 3.10, left panels), being consistent with PPAR δ repression which is evident 3-6 hours after treatment (He *et al.*, 1999). HDAC2 reduction is unlikely the consequence of APC-induced apoptosis since signs of cell death (e.g. rounding-up and detachment) only were seen from 24-36 hrs after ZnCl₂-treatment (Morin *et al.*, 1996; data not shown). Furthermore, if loss of cells was the cause one would also expect effects on HDAC3 and actin controls. These results indicate HDAC2 expression is elevated in APC-mutated HT-29 colon carcinoma cells and is significantly reduced upon restoring wt APC.

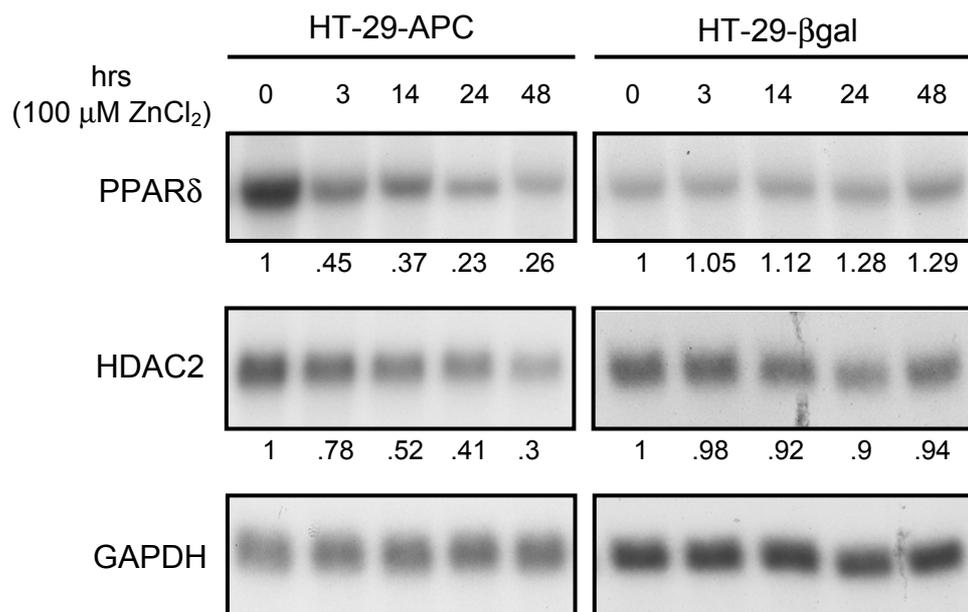


Figure 3.9 Down-regulation of PPAR δ and HDAC2 mRNA expression upon restoring wild-type APC in HT-29 cells. HT-29-APC (zinc-inducible APC expression cell line) and HT-29- β GAL (zinc-inducible β -galactosidase expression clone) cells were treated with 100 μ M ZnCl₂. Poly(A)⁺ RNA preparations (5 μ g) from various time points after treatment were used for Northern blot analysis of PPAR δ and HDAC2. The same blots were re-hybridized with GAPDH probe to control for equal mRNA loading. The numbers below the panels indicate the normalized amounts relative to un-treated samples. Similar results were obtained in two independent experiments.

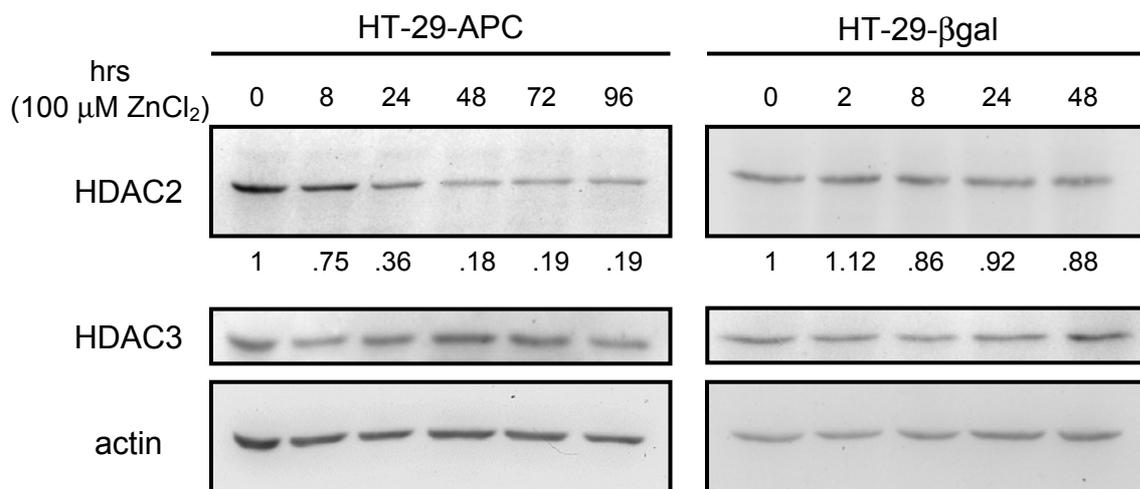


Figure 3.10 The selective reduction of HDAC2 protein levels upon restoring wild-type APC in HT-29 cells. HT-29-APC and HT-29-βGAL cells were treated with 100 μM ZnCl₂. Whole cell extracts from a series of time points after treatment were subjected to Western blot analysis of HDAC2 and 3. Actin signal served as the loading control. The numbers below the panels indicate the normalized amounts related to un-treated samples. Two additional experiments showed similar results.

3.2.2 Elevated HDAC2 expression in colorectal carcinoma tissues

If HDAC2 plays an oncogenic role in colorectal tumorigenesis under the regulation of APC, it could be overexpressed in colon carcinomas, in particular those containing APC mutations or β -catenin mutations. Yang *et al.* (1997) have shown that HDAC2, but not HDAC1 and 3, is overexpressed in a number of carcinoma cell lines including colon cancer cells compared with normal tissues by Northern blot analysis. I investigated HDAC2 protein expression using tissue arrays (BioCat, Heidelberg, Germany) that contain various matched normal and tumor tissues. In gastrointestinal (GI) system, HDAC2 expression is highly elevated in carcinoma region compared to matched normal tissue from the same patient, as shown by immunohistochemistry (Fig. 3.11 and Fig. 3.13). The tumor tissue from a patient with colon adenocarcinoma (mucinous type) demonstrated a high specific HDAC2 staining predominantly in nuclei, whereas the staining in normal colon tissue (in particular mucosa) is very weak (Fig. 3.11). Similar results were also observed in the tissues from a gastric adenocarcinoma (intestinal type) patient (Fig. 3.13). To show the consistency with the proposition that HDAC2 is regulated by the APC/ β -catenin pathway, β -catenin expression was also investigated in serial sections. Both of the carcinomas showed a higher β -catenin expression with substantial staining in membrane and cytoplasm and slight staining in nuclei (Fig. 3.12 and Fig. 3.14). To further evaluate the HDAC2 expression in colorectal carcinomas, a colorectal tissue array (BioCat, Heidelberg, Germany) was used, which contains patient-matched colorectal normal and tumor tissues. Of 46 analyzable sample pairs that showed clear staining, 38 pairs (83%) were with higher HDAC2 staining in carcinoma compared to matched normal tissue (Mengwasser, personal communication).

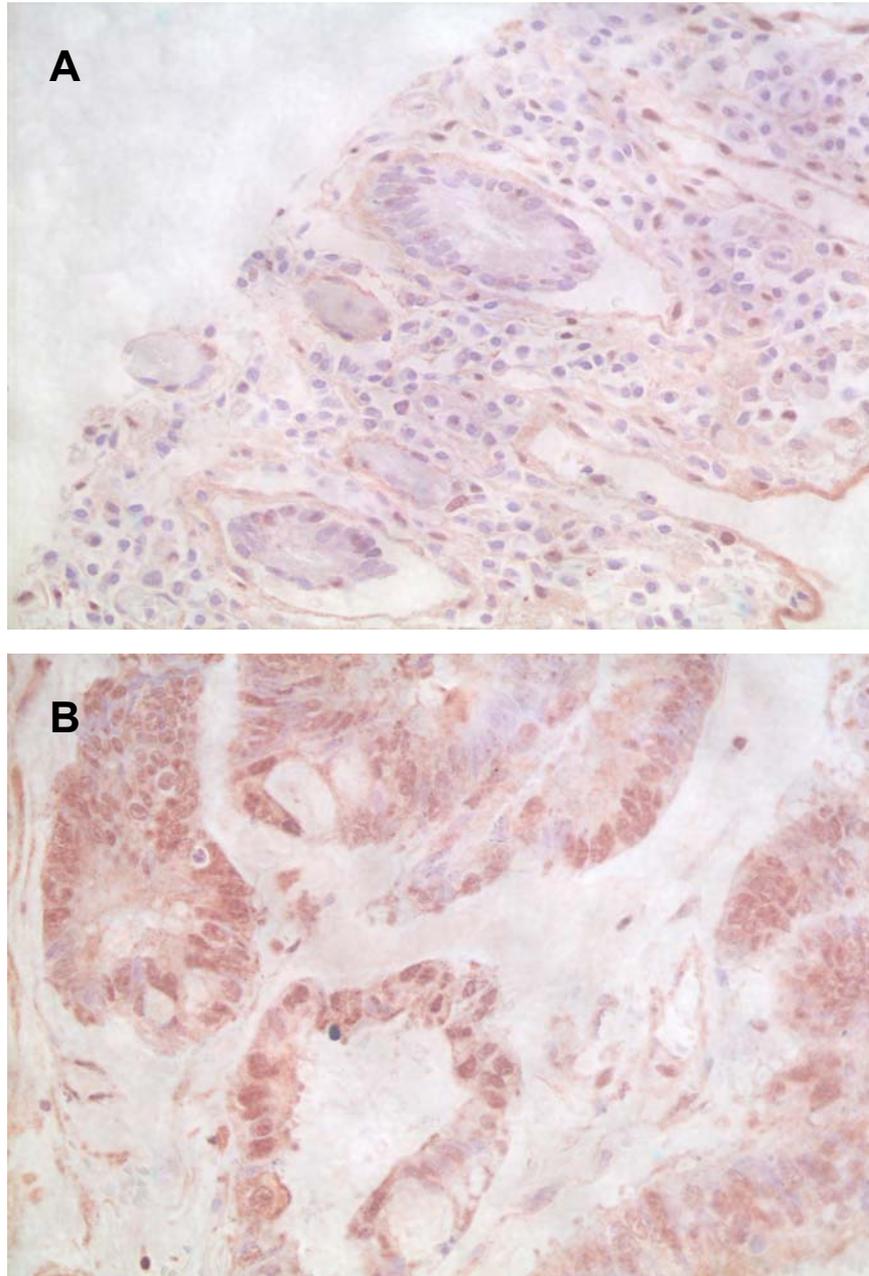


Figure 3.11 Immunohistochemical staining of human colonic tissues with an anti-HDAC2 antibody. Samples are normal colonic tissue (**A**) and colonic adenocarcinoma (mucinous type) tissue (**B**) from the same patient (male, 69). HDAC2 staining (brick red) is predominantly localized to the nucleus. Slides were counterstained with hematoxylin to show the nucleus (light blue). No specific staining was observed in control slides that were stained without primary antibody against HDAC2 (data not shown).

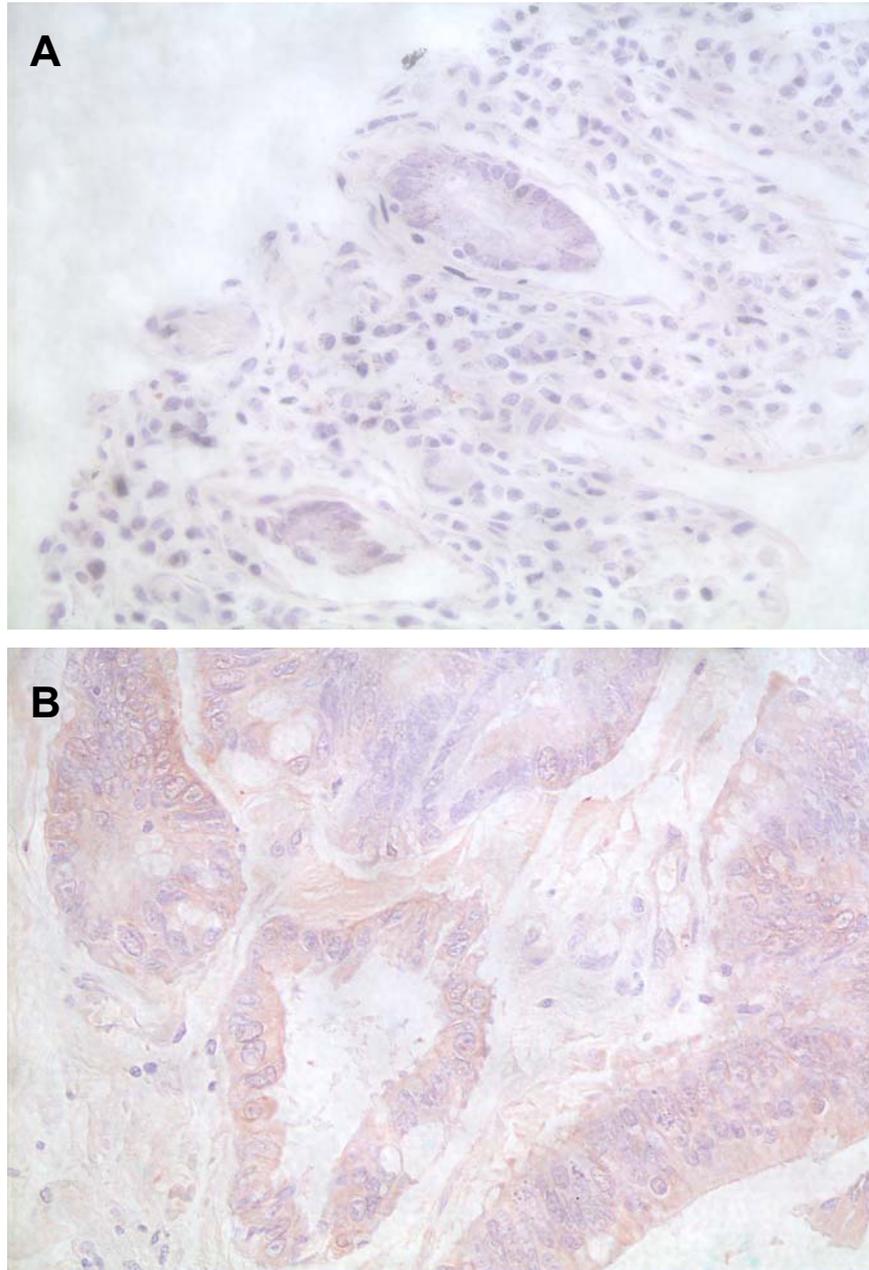


Figure 3.12 Immunohistochemical staining of human colonic tissues with an anti- β -catenin antibody. Samples are normal colonic tissue (**A**) and colonic adenocarcinoma (mucinous type) tissue (**B**) from the same patient (male, 69). Serial sections to those shown in Fig. 3.11 were used. β -catenin staining (brick red) is localized predominantly to membrane and cytoplasm and slightly to nucleus. Slides were counterstained with hematoxylin to show the nucleus (light blue). No specific staining was observed in control slides that were stained without primary antibody against β -catenin (data not shown).

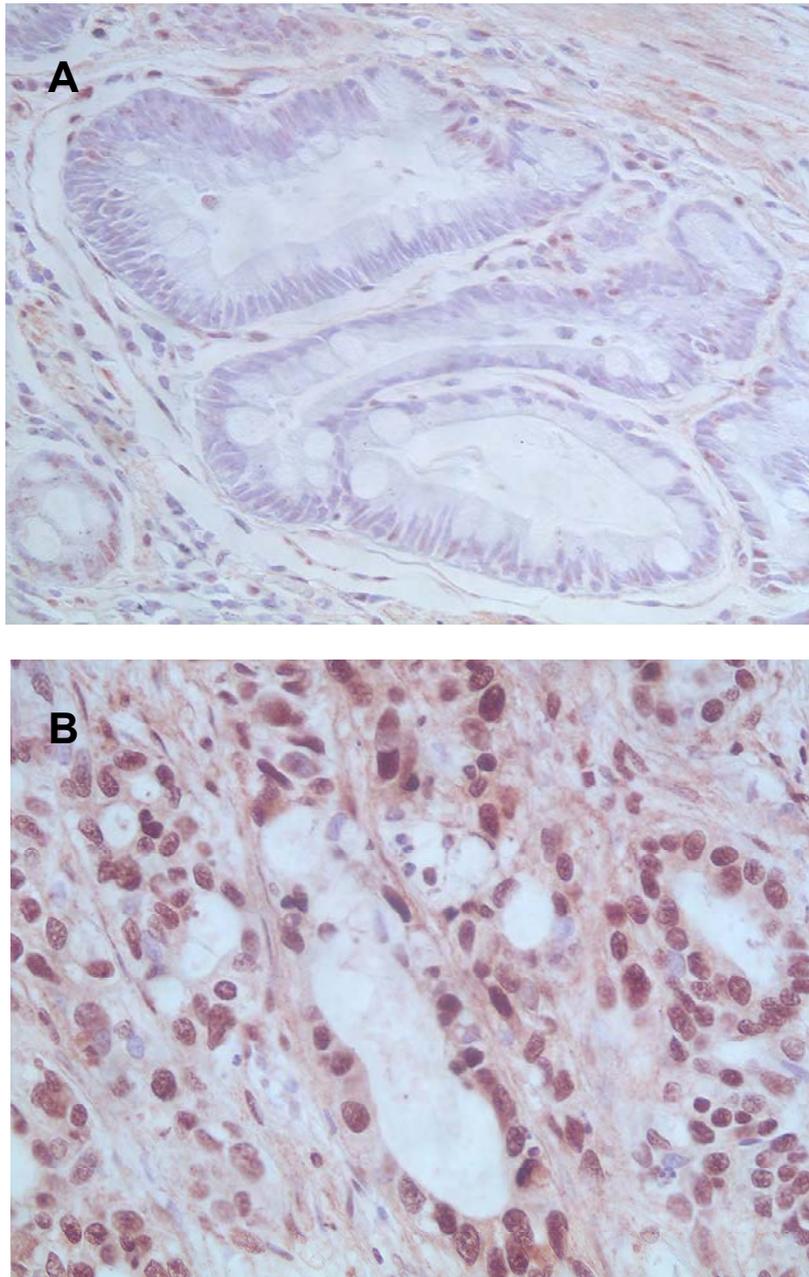


Figure 3.13 Immunohistochemical staining of human gastric tissues with an anti-HDAC2 antibody. Samples are normal gastric tissue (**A**) and gastric adenocarcinoma (intestinal type) tissue (**B**) from the same patient (male, 63). HDAC2 staining (brick red) is predominantly localized to the nucleus. Slides were counterstained with hematoxylin to show the nucleus (light blue). No specific staining was observed in control slides that were stained without primary antibody against HDAC2 (data not shown).

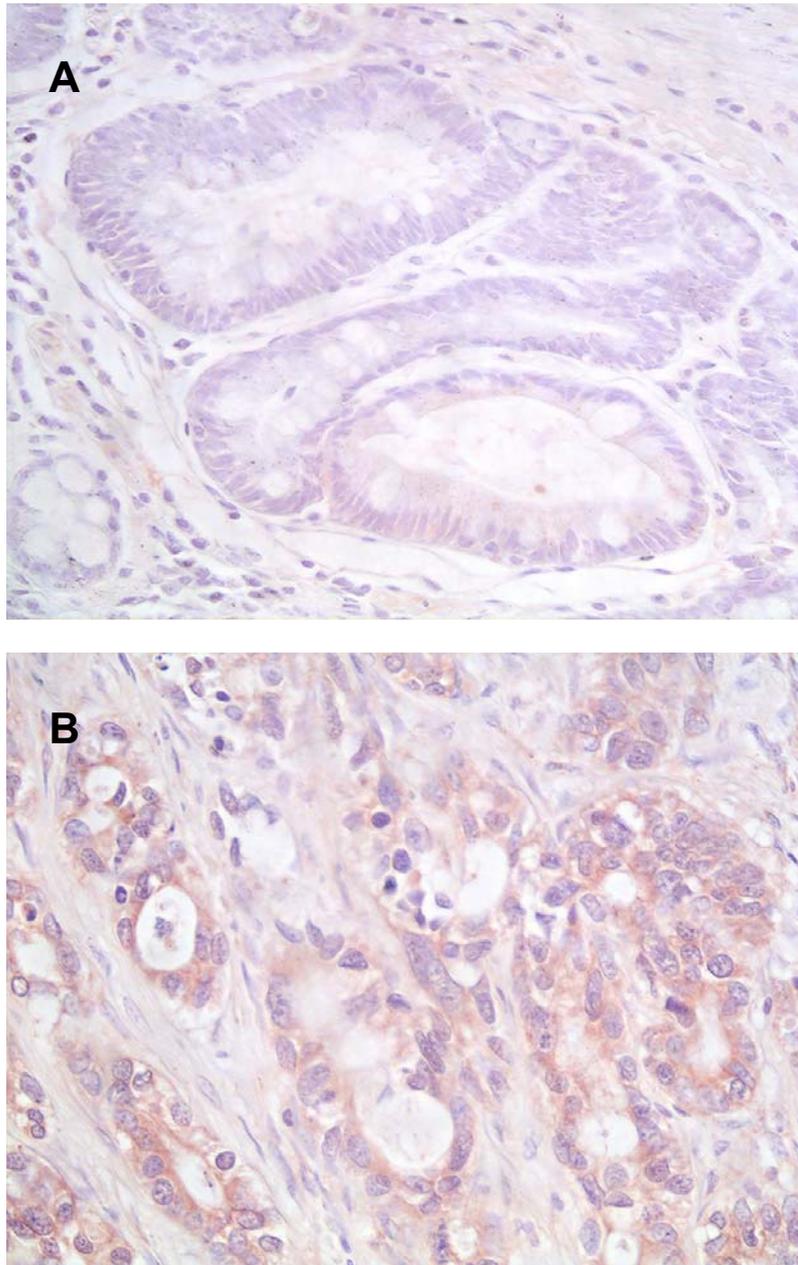


Figure 3.14 Immunohistochemical staining of human gastric tissues with an anti- β -catenin antibody. Samples are normal gastric tissue (**A**) and gastric adenocarcinoma (intestinal type) tissue (**B**) from the same patient (male, 63). Serial sections to those shown in Fig. 3.13 were used. β -catenin staining (brick red) is localized predominantly to membrane and cytoplasm and slightly to nucleus. Slides were counterstained with hematoxylin to show the nucleus (light blue). No specific staining was observed in control slides that were stained without primary antibody against β -catenin (data not shown).

3.2.3 Specific knock-down of HDAC2 by RNAi technology in HT-29 cell line

The reduction of HDAC2 upon restoring wt APC in colonic cancer cells and its overexpression in colorectal tumors suggest a positive role of HDAC2 in the transformed phenotype of colorectal cancer cells. This was firstly tested by a loss-of-function approach with respect to HDAC2. RNA interference (RNAi) technology was selected to knock down the expression of HDAC2 since it was proven to be a very potent and sequence-specific gene-silencing method in mammalian cells (Elbashir *et al.*, 2001; Harborth *et al.*, 2001, Elbashir *et al.*, 2002). The small interfering RNAs (siRNA), in particular 21-nucleotide RNA duplexes are believed to reduce endogenous mRNA levels possibly via RNA destabilization pathways (Bosher and Labouesse, 2000; Elbashir *et al.*, 2002; Hutvagner and Zamore, 2002; Fig. 3.15). The siRNA of HDAC2 was selected to specifically target the sequence corresponding to 96-114 nucleotides of the coding region of human HDAC2 (GCC UCA UAG AAU CCG CAU G) (Fig. 3.15). The non-relevant siRNA, 21-nt RNA duplex that targets firefly luciferase (GL2, Elbashir *et al.*, 2001), was used as a control. Transfections of siRNA were carried out in 24-hr intervals for maintaining cellular siRNA concentration. The effect of HDAC2 RNAi was evaluated by Western blot analysis after transfecting siRNA into HT-29 cells. As shown in Fig. 3.16, HDAC2 protein level was significantly decreased upon transfection with HDAC2 siRNA but not control Luc siRNA. The control siRNA samples and non-transfected samples showed no difference with respect to HDAC2 expression, indicating the non-relevant siRNA and transfection reagent do not influence the experiment. The reduction of HDAC2 expression was detectable at 24 hours after transfection and was most pronounced at 48 and 72 hours, whereas at 96 hours the expression was recovered slightly. HDAC1 and 3, two other class I HDACs, were not affected upon HDAC2 siRNA delivery (Fig. 3.16), suggesting that the HDAC2 RNAi effect is specific. Taken together, the RNAi technology was successfully applied in HT-29 cells to reduce HDAC2 expression efficiently and specifically.

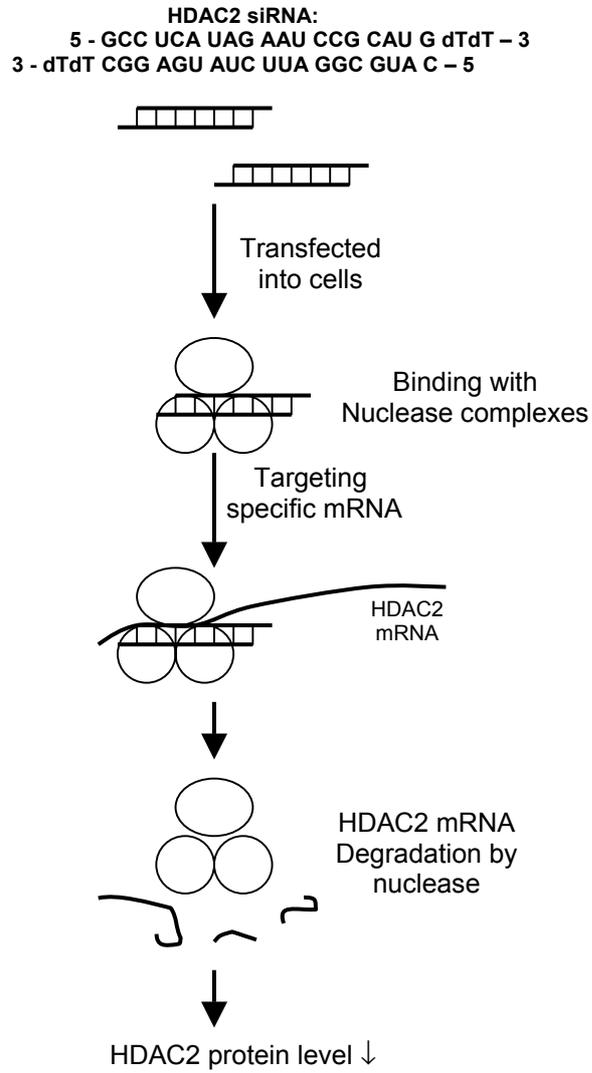


Figure 3.15 The schematic representation of proposed mechanism of RNAi-mediated gene silencing.

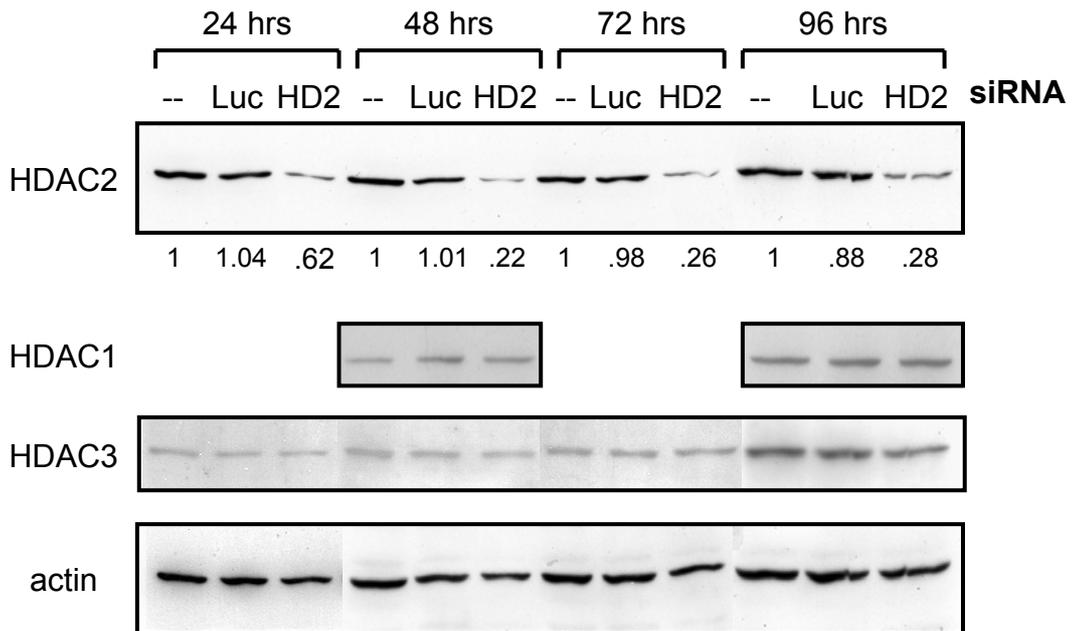


Figure 3.16 Specific and potent HDAC2 reduction by RNAi technology. HDAC2 as well as HDAC1 and 3 protein levels were detected by Western blot analysis after transfection of siRNAs (time course as indicated, --: without transfection, Luc: luciferase siRNA transfection, HD2: HDAC2 siRNA transfection). Actin amounts were determined as loading control. The numbers below the panels indicate relative levels to non-transfected (-) samples upon being normalized to actin signals. Three independent experiments were done with similar results.

3.2.4 Reduction of HDAC2 leads to decreased cell numbers in HT-29 cell cultures

The first consequence observed inhibition after RNAi-mediated HDAC2 reduction in HT-29 cells was a decrease of attached cell numbers. The non-transfected cells and control Luc siRNA-transfected cells displayed no significant difference with respect to growth kinetics, suggesting control siRNA and transfection reagent do not affect the cell growth. In contrast, cultures transfected with HDAC2 siRNA, in which HDAC2 was highly reduced, showed a severe delay in cell number increase with only less than half of the cell counts of control samples at 72 and 96 hours post-transfection (Fig. 3.17 A). Another observation was that more floating cells were found in HDAC2 siRNA transfected samples and the ratio of floating and attached cells was significantly higher in comparison with control samples (Fig. 3.17 B). Interestingly, similar phenomena were also observed in HT-29-APC cells when wt APC was expressed upon zinc-exposure, and the floating cells were shown to be apoptotic cells (Morin *et al.*, 1996). These results suggest that HDAC2 is critical for HT-29 cell survival and/or growth, supporting its role in maintaining critical aspects of the transformed phenotype.

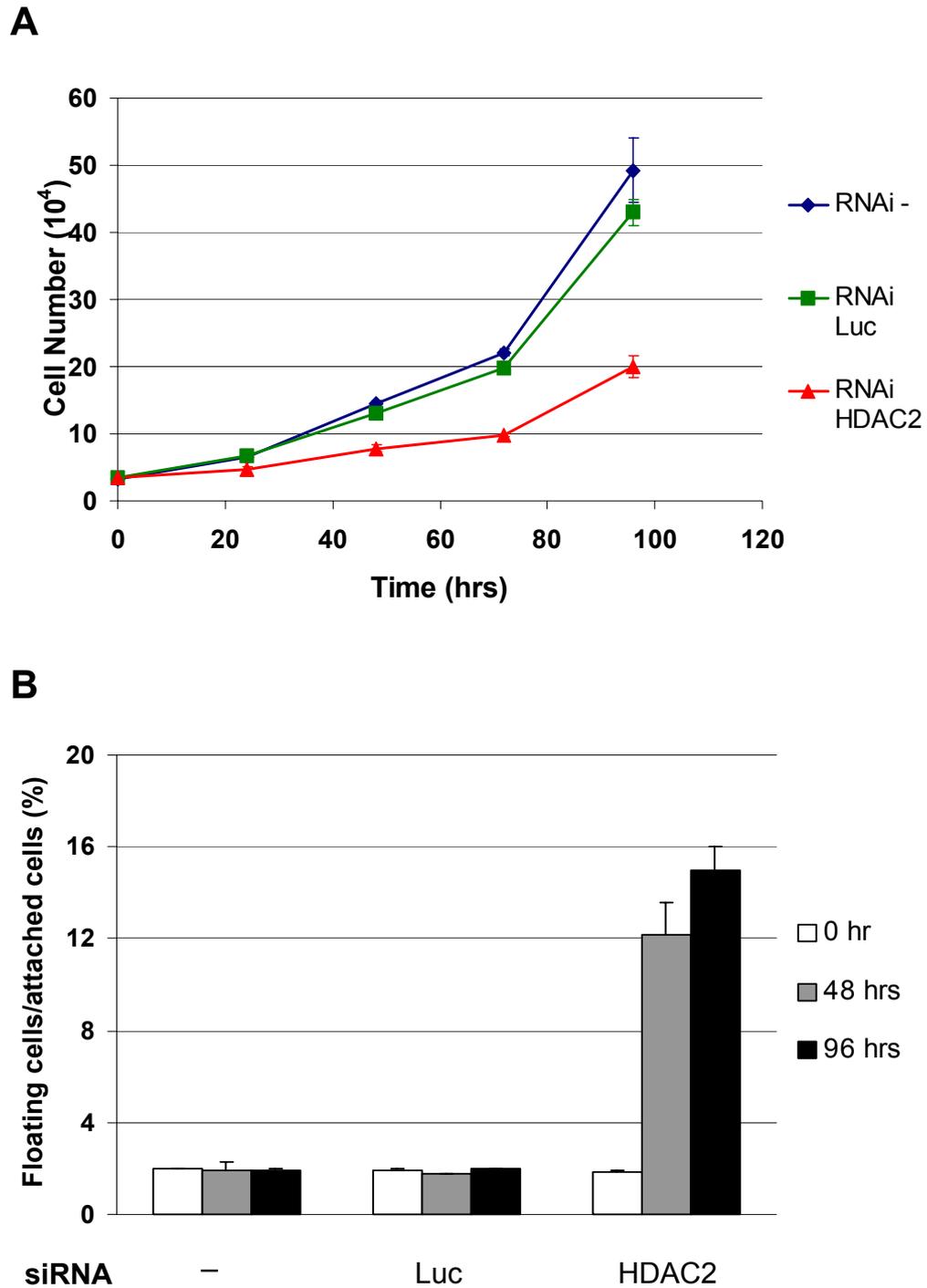


Figure 3.17 Reduction of cell number upon RNAi-induced HDAC2 reduction in HT-29 cells. (A) Accumulation of HT-29 cells was determined by counting attached cells at various time points (0-96 hrs) after siRNA transfection. Values are means \pm SD of duplicate samples. (B) The ratios of floating to attached cell counts were determined at indicated time points after transfection. -: without transfection, Luc: luciferase siRNA control transfection, HDAC2: HDAC2 siRNA transfection. One representative out of three similar experiments is shown.

3.2.5 Reduction of HDAC2 does not affect the cell cycle progression of HT-29 cells

Accumulation of cells per culture is the overall effect of cell proliferation and cell death (apoptosis). HDAC inhibitors induce cell cycle arrest at G1 or G2 phase in several cancer cell lines, possibly via regulating the expression of cell cycle regulators like p21 and p27. HDAC2 has been shown to be associated with pRb and E2F that regulate cell cycle progression and cell proliferation (Wang *et al.*, 2001). Thus, I firstly tested the possibility that the cell number decrease is due to cell cycle arrest upon RNAi-mediated HDAC2 reduction. However, this assumption could not be proven since there was no change between HDAC2 siRNA transfected and control cells with respect to the cell fractions in G1 and S/G2 phases, indicating cells were not blocked in cell cycle. The only difference observed was an increased cell population with sub-G1 DNA content, which often indicates apoptotic cells (Wyllie, 1995) (Fig. 3.18). Interestingly, similar effects were also found in HT-29-APC cells upon inducing wt APC expression (Morin *et al.*, 1996; data not shown). The observation suggests that HDAC2 reduction does not influence the cell cycle progression of HT-29 cells, but rather might induce apoptosis.

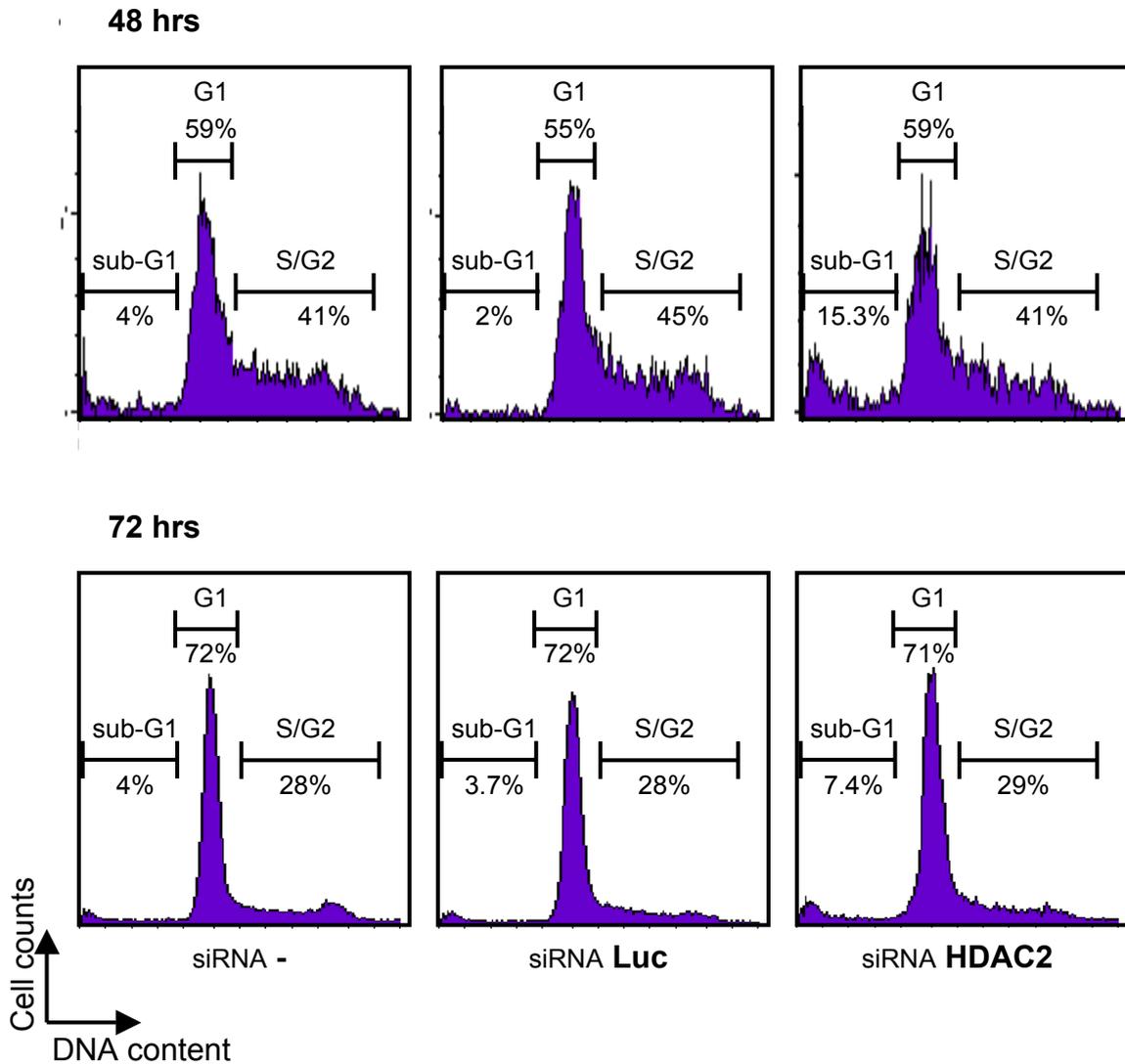


Figure 3.18 HDAC2 reduction does not influence cell cycle progression in HT-29 cells. At indicated time points after siRNA transfection, both attached and floating cells were harvested and stained with Hoechst dye. Cell cycle profiles were analysed with the flow cytometer and CellQuest pro software. The percentages of sub-G1 fraction (cells with lower DNA content) are ratios of sub-G1 cell counts to total cell counts (sub-G1, G1 and S/G2). The percentages of cells in G1 (or S/G2) fraction represent ratios of cell counts in G1 (or S/G2) phase to total cell counts in G1 and S/G2 phases. One representative out of three similar experiments is shown.

3.2.6 Reduction of HDAC2 increases apoptosis in HT-29 cells

More floating cells and increased sub-G1 population strongly suggested elevated cell death after HDAC2 down-regulation, which could be a cause of cell number decrease in cultures. Apoptosis was assessed by two DNA fragmentation-based assays to confirm the increased rate of apoptosis upon reduction of HDAC2 levels. DNA staining of the floating and attached cells with DNA-binding Hoechst dye was used to distinguish apoptotic and living cells by microscopic evaluation. The cells containing condensed or fragmented nuclei were counted as apoptosis cells (Fig. 3.19 A). As shown above, HDAC2 down-regulation resulted in more floating cells, and the Hoechst staining demonstrated almost all of the floating cells were undergoing apoptosis from both HDAC2 siRNA transfected and control samples (Fig. 3.19 A, data not shown). The ratio of floating (apoptotic) cells and total cells was about 10-15% upon HDAC2 reduction whereas it was much lower (2-3%) under control conditions (Fig. 3.19 B). In attached cells, the ratio (attached apoptotic cell counts/total cell counts) was also much higher (about 8%) upon HDAC2 reduction than that of control samples (about 2%) (Fig.3.19 B). Hoechst staining provided evidence for the proposition that knock down of HDAC2 expression leads to an elevated apoptosis in HT-29 cells.

The Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) was further used to confirm the apoptosis event. This kit provides a quantitative apoptosis assay by determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) generated during the apoptotic process. For samples used in this assay, the OD values directly correlate with the amounts of mono- and oligonucleosomes in the cytoplasm, namely, the degrees of apoptosis. The assay again demonstrated that the floating cells were indeed undergoing apoptosis since they showed a much higher OD value compared to similar amounts of attached cells (Fig. 3.20 A). To evaluate the overall apoptosis, both floating and attached cells were harvested for this apoptotic assay. Compared to control samples, the overall apoptosis was

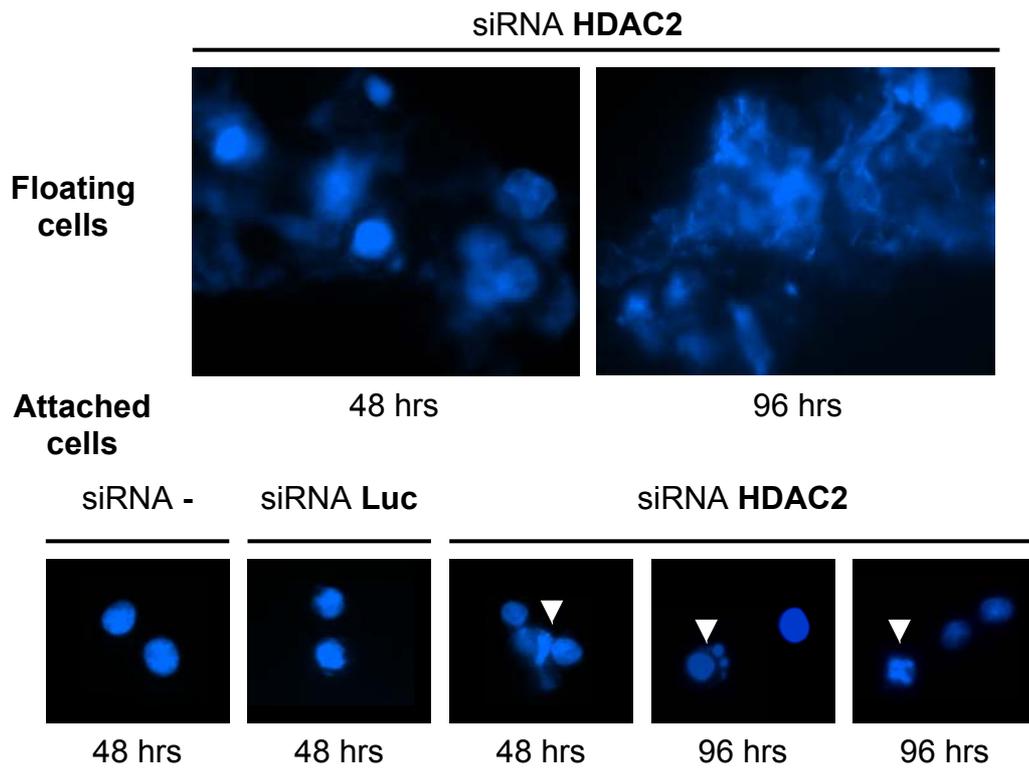
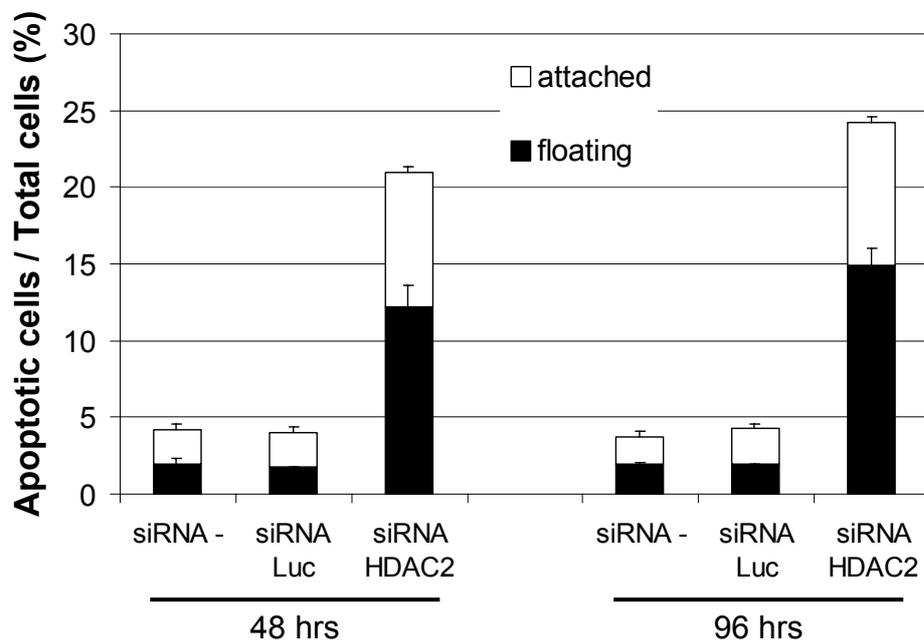
A**B**

Figure 3.19 RNAi-induced HDAC2 reduction enhances apoptosis in HT-29 cells. (A) Fluorescence microscopy of cells stained with Hoechst dye at various time points after siRNA transfections as indicated. The apoptotic nuclei (fragmentation and condensation) were found in virtually all floating cells and some attached cells (indicated by arrows). **(B)** The bars represent ratios of apoptotic cell counts versus total (floating and attached cells of a culture) cell counts (means \pm SD from two independent experiments).

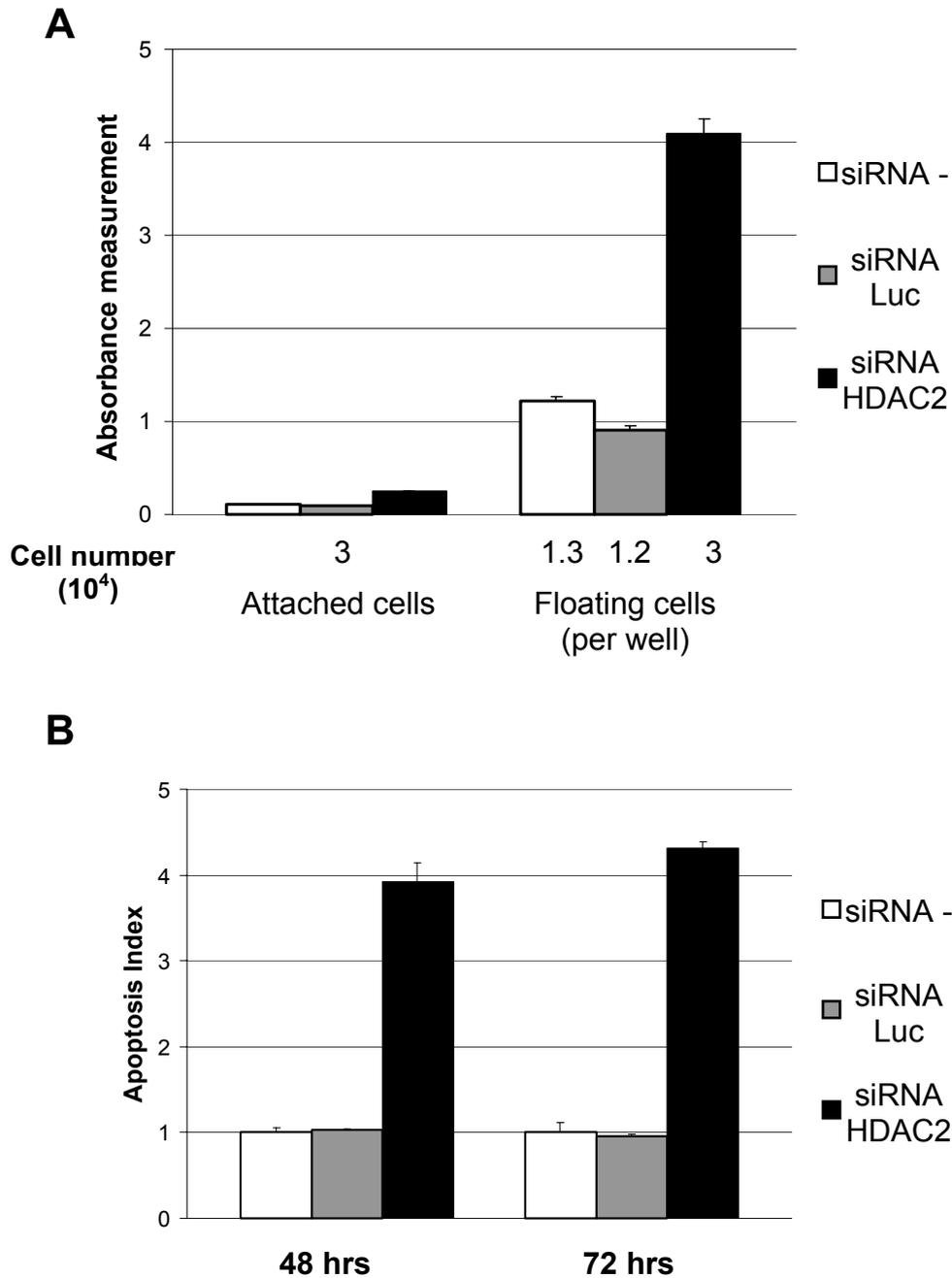


Figure 3.20 RNAi-induced HDAC2 reduction enhances apoptosis in HT-29 cells (assessed by Roche ELISA kit). The Cell Death Detection ELISA^{PLUS} kit (Roche) was used for quantitative apoptosis assay. The amount of chromophoric substrate produced in a chromogenic reaction catalyzed by anti-DNA antibody conjugated peroxidase was measured photometrically at 415 nm. Absorbance measurement correlates with the amount of apoptosis-associated cytoplasmic mono- and oligonucleosomes present in the cell extract. **(A)** Absorbance measurements (corrected for background value) were determined for extracts from cells 48 hrs after siRNA transfection (means \pm SD from duplicate samples). 3×10^4 attached cells or total floating cells of a culture (cell number indicated) were used for the assay. **(B)** Extracts from 1×10^5 out of total cells (floating and attached) were subjected to the assay. Apoptosis indexes represent relative absorbances from transfected samples (Luc or HDAC2) to those from un-transfected samples (-). Values are means \pm SD from duplicate samples. Three independent experiments were performed with similar results.

increased by about 3-fold in HT-29 cells transfected with HDAC2 siRNA (Fig. 3.20 B).

These two different apoptosis assays clearly indicated that RNAi-induced HDAC2 reduction increases apoptosis in HT-29 cells. Therefore, the crucial role of HDAC2 for preventing apoptosis and maintaining transformed phenotype of HT-29 cells was coming into view. Moreover, HDAC2-deficiency induced apoptosis could be an explanation of the impaired accumulation of cell number after HDAC2 siRNA transfection.

3.2.7 HDAC2 reduction has a moderate effect on HT-29 cell differentiation

Besides cell cycle progression and apoptosis, another important cell process is differentiation. HDACs have been shown to be involved in cell differentiation, e.g. aberrant recruitment of HDAC activity blocks normal hematopoietic cell differentiation in APL and AML patients, and HDAC2 is required for obstructing the neurite differentiation upon association with coREST (Ballas *et al.*, 2001). Potential differentiation of HT-29 cells upon HDAC2 down-regulation was assessed by measuring the cellular alkaline phosphatase (ALP) activity which is known as a differentiation maker of colorectal cells (Matsumoto *et al.*, 1990; Hinnebusch *et al.*, 2002). The ALP activities from whole cell lysates of HDAC2 siRNA transfected samples were about 50% higher than those of control samples (Fig. 3.21 A), indicating that HDAC2 reduction has at least a mild effect on HT-29 cell differentiation. In HT-29-APC cells zinc-induced wt APC expression and subsequent reduction of HDAC2 increased cellular ALP activity slightly more efficiently (about 2.2-fold, Fig. 3.21 B). These data suggested that HDAC2 reduction plays a role in the differentiation of HT-29 cells and is likely to account at least partially for APC-dependent cell differentiation.

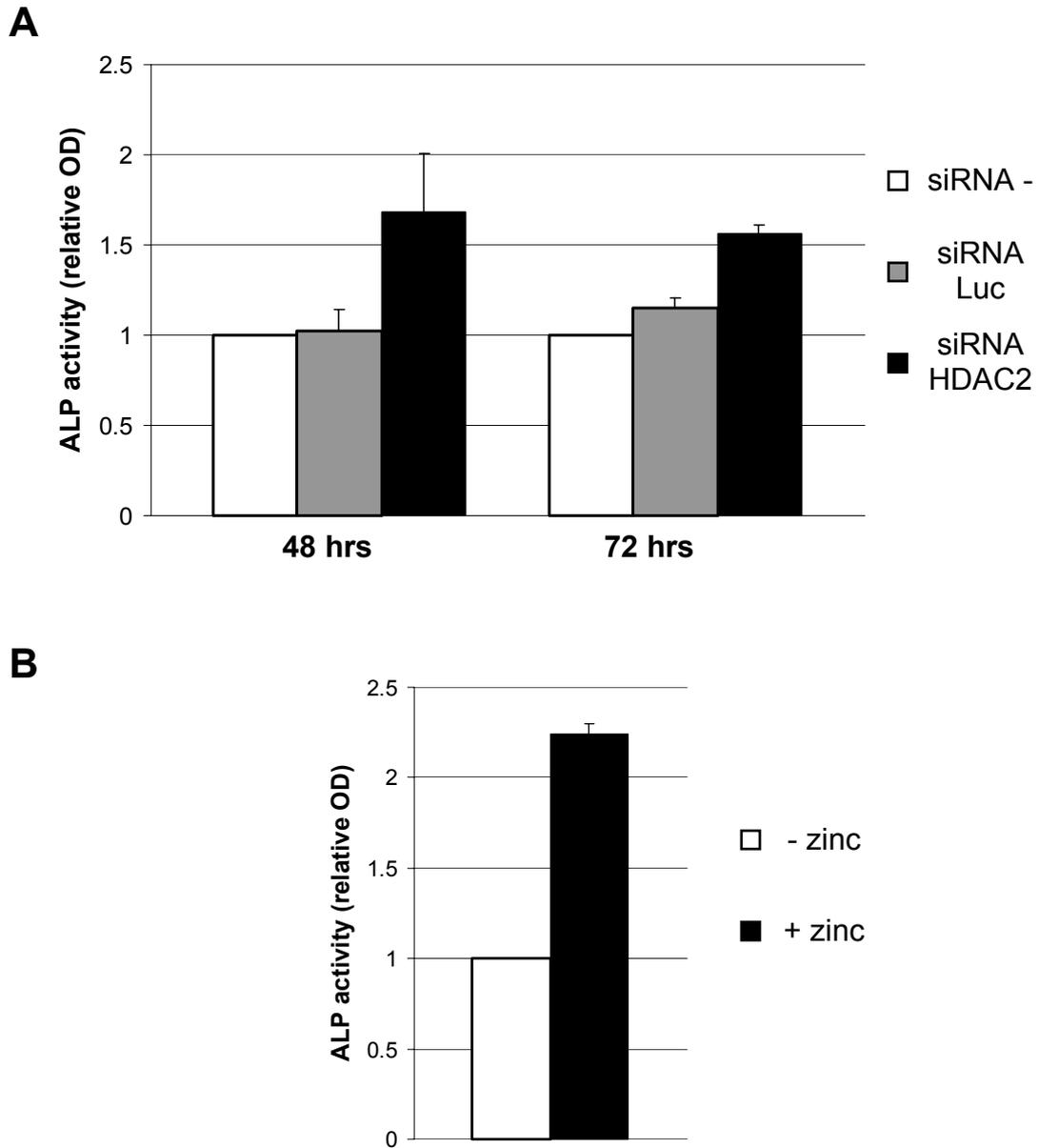


Figure 3.21 Alkaline phosphatase (ALP) activity in HT-29 cells. (A) ALP activities in whole cell lysates were determined at indicated time points after siRNA transfection. Values are relative ODs to un-transfected samples (siRNA -) (means \pm SD from three independent experiments carried out in duplicate). **(B)** ALP activities from HT-29-APC cells (48 hrs 100 μ M ZnCl₂-treated and non-treated) are shown as relative ODs to untreated samples. Values are means \pm SD from two independent experiments performed in duplicate each.

3.2.8 Ectopic HDAC2 expression antagonizes the APC-induced apoptosis

A critical role of HDAC2 in controlling apoptosis of HT-29 cells was so far supported by evidence from loss-of-function conditions. To confirm this role of HDAC2 by a gain-of-function analysis, HDAC2 was ectopically overexpressed in HT-29-APC cells in which endogenous HDAC2 was repressed by wt APC expression. Mouse HDAC2 (with 93% identity to human HDAC2) or the empty expression vector was co-transfected with a green fluorescent protein (GFP) expression vector into HT-29-APC cells. Then wt APC was induced by ZnCl₂-treatment in order to down-regulate endogenous HDAC2 expression (Fig. 3.9 and 3.10) and the cells were in turn undergoing apoptosis. Ectopically expressed HDAC2 should rescue the APC-induced cell death if HDAC2 reduction is crucial for APC-induced apoptosis. Consequently the GFP-positive cells, which are also likely to express HDAC2 ectopically, should be survival and be enriched upon zinc-treatment in HDAC2/GFP co-transfected samples. As shown in Fig. 3.22, the ratios of GFP-positive cells increased by about 50% at 48 hrs and by 100% at 72 hrs after ZnCl₂-treatment in HDAC2/GFP co-transfected samples, compared to untreated cells. Moreover, no significant enrichment of GFP-positive cells was observed by the same ZnCl₂-treatment in control (empty vector/GFP) transfections. This experiment suggests that HDAC2 overexpression antagonizes, at least partially, APC-mediated HT-29 cell apoptosis.

3.2.9 Summary of this section

The results in this section reveal a crucial role of HDAC2 in preventing apoptosis and maintaining transformed phenotype of colorectal cancer cells. HDAC2 expression is found to be elevated in colorectal cancer cells (HT-29 cells) and in most tested colorectal carcinomas, and restoring wt APC expression down-regulates HDAC2 expression in HT-29 cells. HDAC2 reduction by RNAi technology arrests accumulation of HT-29 cell number probably due to the significantly elevated apoptosis. HDAC2 down-regulation

also moderately induces HT-29 cell differentiation, but does not affect cell cycle progression. Moreover, APC-mediated apoptosis in HT-29 cells could be rescued upon ectopic overexpression of HDAC2.

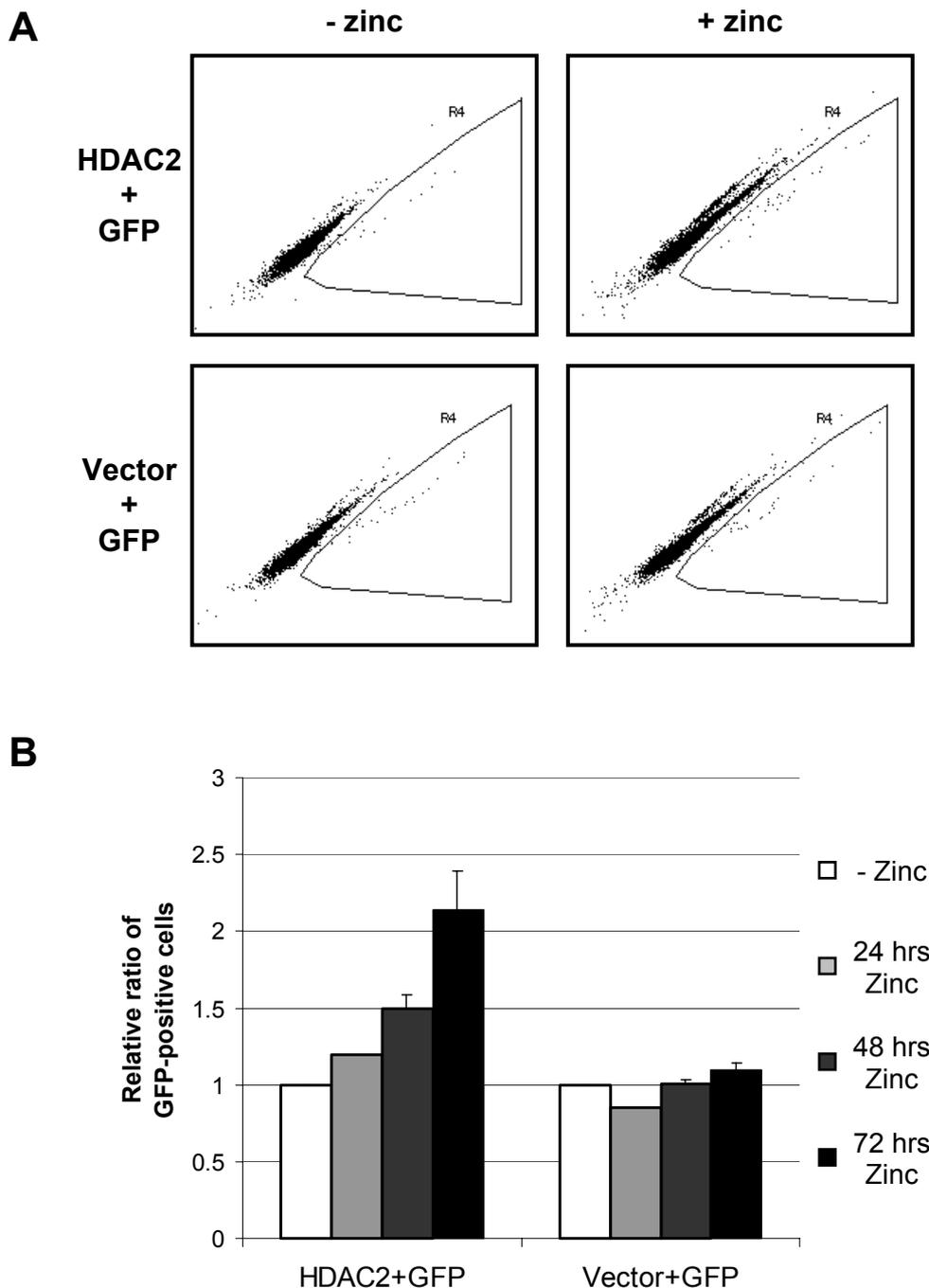


Figure 3.22 Ectopic overexpression of HDAC2 antagonizes APC-induced apoptosis in HT-29 cells. (A) The pCMV-GFP (GFP expression vector) was cotransfected with the murine HDAC2 expression vector pCMV-mRPD3-2.1(HDAC2+GFP) or the control empty vector pcDNA3.1(+) (Vector+GFP) into HT-29-APC cells. For ZnCl₂-treated (+zinc) samples, 12 hrs after transfection ZnCl₂ (f.c. 100 μM) was added to induce wt APC expression. Attached cells were harvested 24, 48 and 72 hrs after ZnCl₂-treatment and analyzed by FACS. A representative experiment (72 hrs treatment) is shown. High fluorescent GFP-positive cells were indicated. (B) Bars represent relative ratios of GFP-positive cells to ZnCl₂-untreated samples of corresponding time points. Values are means ± SD of two independent experiments performed in duplicate each. Actual ratios of GFP-positive cells were in the range of 0.5-4% for all samples. The data were analyzed with CellQuest pro software.

3.3 VPA selectively inhibits a subset of HDAC activity

Another question, which is deduced from the initial observation that VPA activates PPAR δ -dependent gene expression, to be addressed was how VPA activates PPAR δ -dependent transcription. Failure to show that VPA could bind to PPAR δ argued against VPA itself serving as a ligand to PPAR δ (Lampen *et al.*, 2001, Göttlicher, personal communication). Whether metabolites of VPA could be PPAR δ -binding ligands is unknown. Besides ligand activation, an alternative activation (or de-repression) model for some NRs like PPAR δ is inhibition of HDAC activity associated with corepressors (Fig. 1.4). The assumption that VPA activates PPAR δ -dependent transcription by inhibiting HDACs was tested in this section.

3.3.1 VPA inhibits N-CoR-associated HDAC activity *in vitro*

N-CoR is a corepressor for NRs and other transcription factors which has been shown to associate with several known HDACs (Heinzel *et al.*, 1997; Kao *et al.*, 2000; Huang *et al.*, 2000; Wen *et al.*, 2000). The immunoprecipitated N-CoR from F9 and HEK293T cell extracts was used as an enzymatic source for *in vitro* HDAC assay to test whether VPA inhibits HDAC activity directly. Already at a concentration of 0.5 mM, VPA inhibited N-CoR-associated HDAC activity almost as efficiently as TSA (300 nM), a well-established HDAC inhibitor (Yoshida *et al.*, 1990) (Fig. 3.23 A). In the presence of 5 mM VPA, HDAC activity was blocked completely. To check which HDAC(s) was associated with N-CoR in this assay, Western blot analysis was carried out, which indicated that significant amounts of HDAC3 was co-immunoprecipitated with N-CoR whereas HDAC1 and 2 were poorly associated. This observation suggests that HDAC3 contributed to the enzymatic activity associated with N-CoR (Fig. 3.23 B). It has been reported that HDAC3 activity is significantly augmented upon binding of HDAC3 to N-CoR (Wen *et al.*, 2000; Guenther *et al.*, 2001). Thus, the HDAC inhibition of VPA could be due to a simple disruption of N-CoR/HDAC3 complexes. To rule

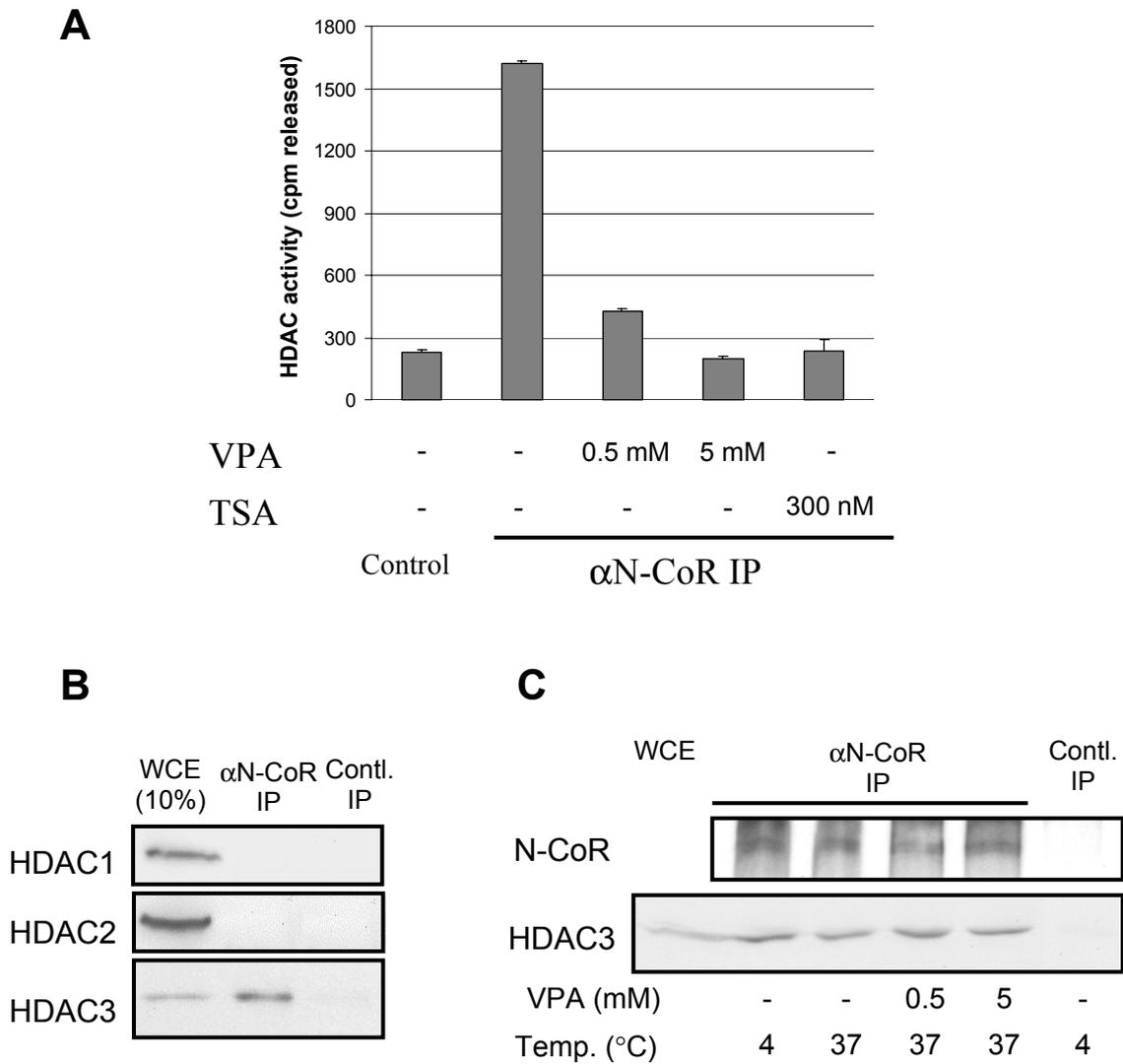


Figure 3.23 VPA inhibits N-CoR-associated HDAC activity *in vitro*. (A) The HDAC activity of N-CoR immunoprecipitates (α N-CoR IP) from F9 cells in the absence and presence of VPA and TSA. Precipitations with pre-immune serum served as the control. Values are means \pm SD of three measurements. One representative of four similar experiments is shown. (B) HDAC(s) associated with the N-CoR IP were detected by Western blot analysis. WCE, whole cell extract. (C) Stability of the N-CoR/HDAC3 complex in the presence of VPA was tested under the conditions of HDAC assay (37°C, 90 min). N-CoR IPs were incubated in the absence and presence of VPA at 37°C for 90 min, the same conditions for HDAC assay. Beads were then washed and detected for the amount of HDAC3 by Western blot analysis (bottom). Equal amount of N-CoR in corresponding IP was shown by Western blot analysis (top). Precipitations with pre-immune serum served as the control.

this out, the N-CoR immunoprecipitate was incubated under conditions similar to those of the HDAC assay to evaluate whether HDAC3 dissociates from the N-CoR complex. Western blot analysis clearly showed that VPA-treatment of the immunoprecipitates *in vitro* did not affect the amount of HDAC3 that was associated with N-CoR (Fig. 3.23 C), suggesting that HDAC inhibition is most likely due to direct effects on HDACs rather than disintegration of the complex.

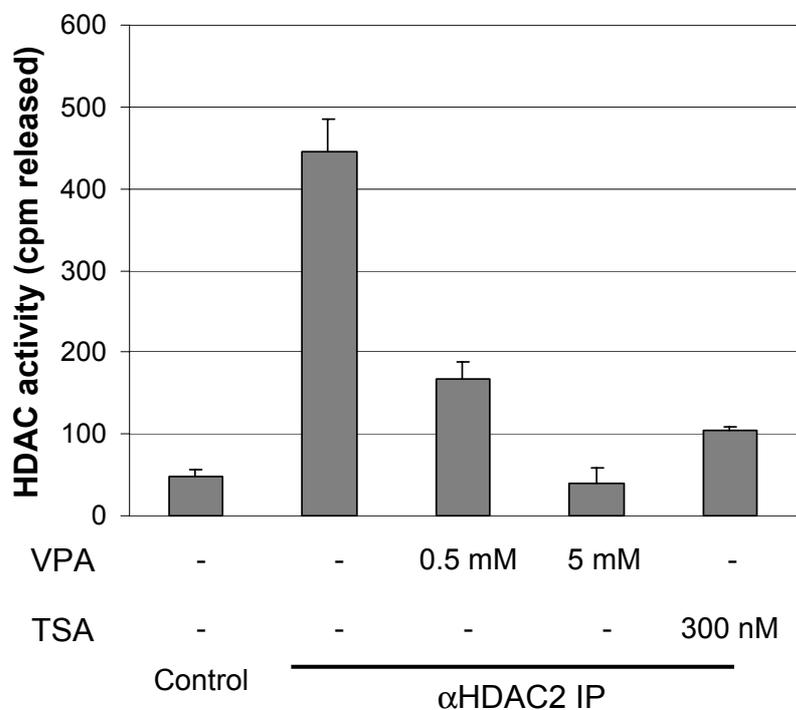
3.3.2 VPA inhibits HDAC2 activity *in vitro*

The direct inhibitory effect on HDAC of VPA was further confirmed using HDAC2 immunoprecipitates for the HDAC assay (Fig. 3.24 B). Again VPA efficiently inhibited HDAC2 activity although slightly higher dose appeared to be required (Fig. 3.24 A).

3.3.3 A VPA-insensitive HDAC subset is abundant in F9 cells

A significant difference between F9 and HEK293T cells was found when HDACs in N-CoR-depleted extracts were tested for susceptibility to VPA, suggesting that VPA could be a selective inhibitor for a subset of HDAC activity. The N-CoR-associated HDAC activity from both cell lines was efficiently inhibited at 0.5 mM VPA (Fig. 3.23 A, Fig. 3.25 A). In contrast, those HDACs which remain in the N-CoR depleted supernatant of F9 cells were inhibited by less than 40% even in the presence of 5 mM VPA, whereas the activity in N-CoR depleted HEK293T cell extracts was inhibited by VPA approximately as efficiently as that in the precipitate (Fig. 3.25 A). Nevertheless, TSA at 300 nM was sufficient to inhibit the HDAC activities from all preparations without discrimination (Fig. 3.25 A). Efficiency of depletion was assessed by Western blot analysis of the immunoprecipitates and supernatants (Fig. 3.25 B). This finding indicated that a VPA-insensitive HDAC subset is abundant in F9 cells. It is likely that the spectrum of HDACs that are not tightly associated with N-CoR differs between the two cell lines and not all HDAC isoforms and/or complexes may be inhibited with equal efficiency by VPA.

A



B

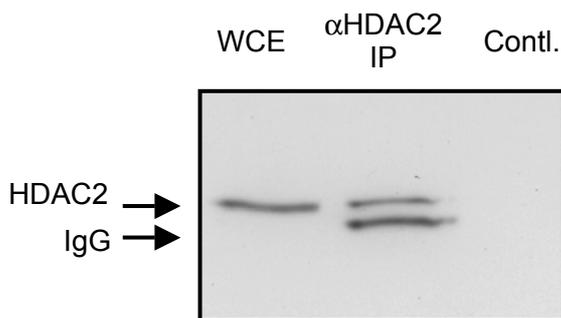


Figure 3.24 VPA inhibits the activity of HDAC2 immunoprecipitate from F9 cells. (A) HDAC activity of HDAC2 Immunoprecipitate (α HDAC2 IP) was determined in the absence and presence of VPA and TSA. Values are means \pm SD of three measurements. One representative of three similar experiments is shown. **(B)** Western blot analysis of the HDAC2 IP used for the HDAC assay is shown. Control (contl.) samples were precipitated without a primary antibody.

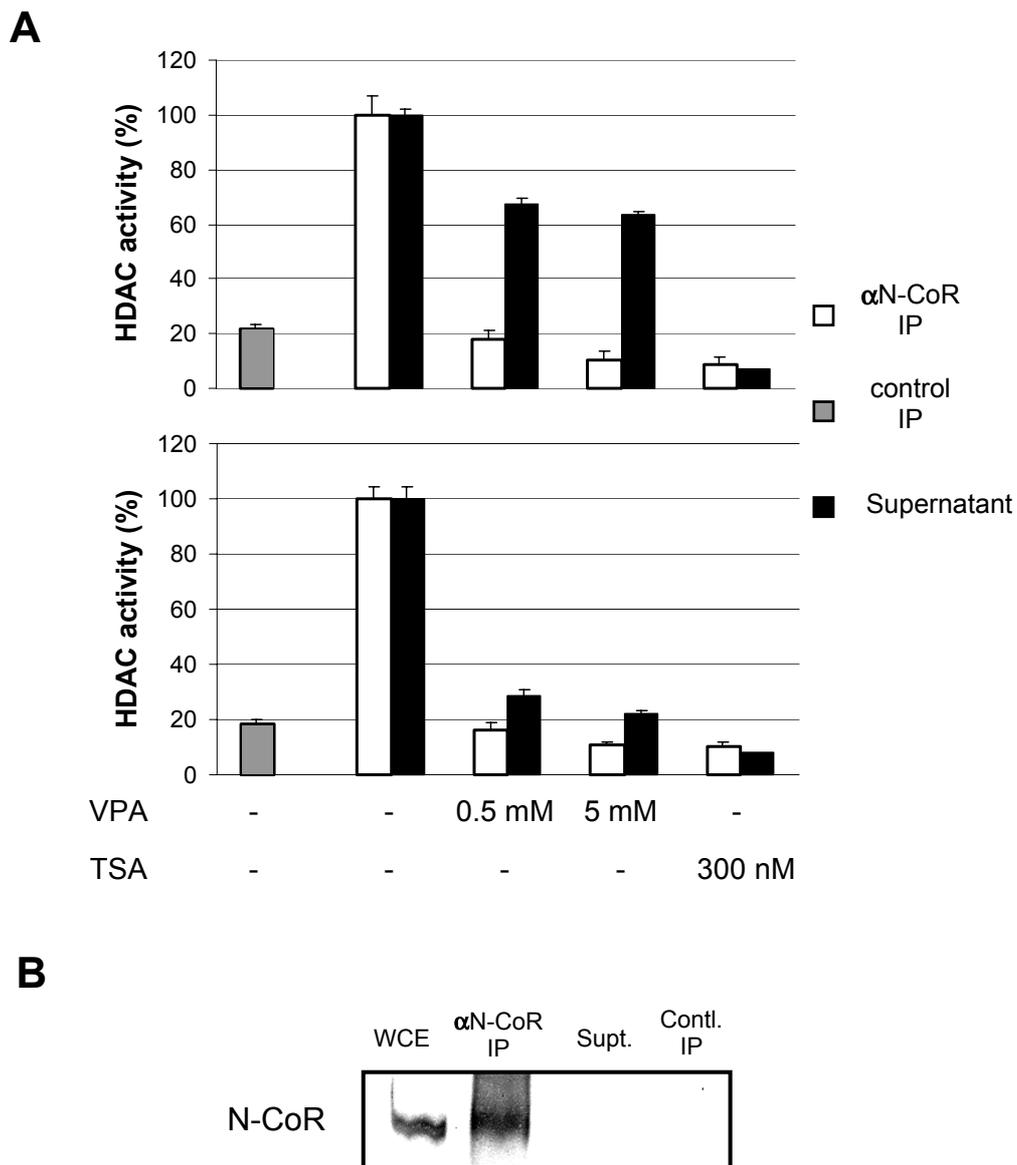


Figure 3.25 Differential inhibitory effects of VPA on HDAC activities in N-CoR immunoprecipitates and N-CoR-depleted extracts from F9 cells. (A) HDAC activity was determined in N-CoR immunoprecipitates (α N-CoR IP) and N-CoR-depleted extracts (Supernatant) from F9 cells (upper panel) and HEK293T cells (lower panel) in the presence or absence of HDAC inhibitors as indicated. Values are means \pm SD of three measurements. Similar results were obtained in two additional experiments. **(B)** Efficiency of N-CoR depletion was assessed by Western blotting for N-CoR in the IP pellet as well as in equivalent amounts of whole cell extracts before (WCE) and after (Supt.) depletion. The experiment with F9 cells is shown.

3.3.4 VPA preferentially inhibits class I HDACs

I further tested which HDAC isoform/complexes are resistant to VPA by determining IC_{50} for distinct HDAC complexes. *In vitro* translated HDACs could not be used as a source for the enzymes since no specific activity was achieved so far (Miska *et al.*, 1999). I considered immunoprecipitation of endogenous HDACs that precludes potential artifact from overexpression of recombinant HDACs. It can, however, not be excluded that even the precipitates obtained with antibodies against individual HDACs contain additional HDAC isoforms, e.g. bound via corepressors. To circumvent this problem, cell extracts were firstly immuno-depleted for all those HDACs and corepressor complexes efficiently inhibited by VPA (IC_{50} -concentrations below 1 mM), e.g. N-CoR, mSin3, and HDACs 1-3 (Fig. 3.26 A) (data in this section; Göttlicher *et al.*, 2001; Phiel *et al.*, 2001). Class II enzymes were focused on apparently. Immuno-detection and precipitation of HDACs 5 and 6 were possible from depleted F9 but not depleted HEK293T cell extracts (Fig. 3.26 B). This finding suggests that in HEK293T cells HDACs 5 and 6 are quantitatively bound in corepressor complexes with other HDACs whereas in F9 cells at least after cell lysis a substantial fraction of HDACs 5 and 6 is not associated with corepressors and class I HDACs.

Then the sensitivity of class II enzymes HDAC5 and 6 to VPA was investigated. The IC_{50} -concentrations for HDAC5 and 6 precipitated from the depleted F9 cell extracts were determined as 2.8 and 2.4 mM VPA, respectively (Table 3.1). Consistent with the deficiency of HDAC5 and 6 in the depleted extracts, the specific HDAC5 and 6 activities were very low in the precipitates from depleted HEK293T cell extracts. Thus, HDAC 5 and 6 could account for the HDAC activity in N-CoR-depleted F9 cell extract that is poorly inhibited by VPA. The apparent IC_{50} -concentrations for VPA are lower if HDACs 5 or 6 are precipitated from whole cell extracts (1.8 or 1.5 mM for HDACs 5 or 6 from F9 cells respectively, Table 3.1). This is consistent with the interpretation that in whole cell extracts a significant fraction of HDACs 5 and 6 exists as components of corepressor complexes together with other

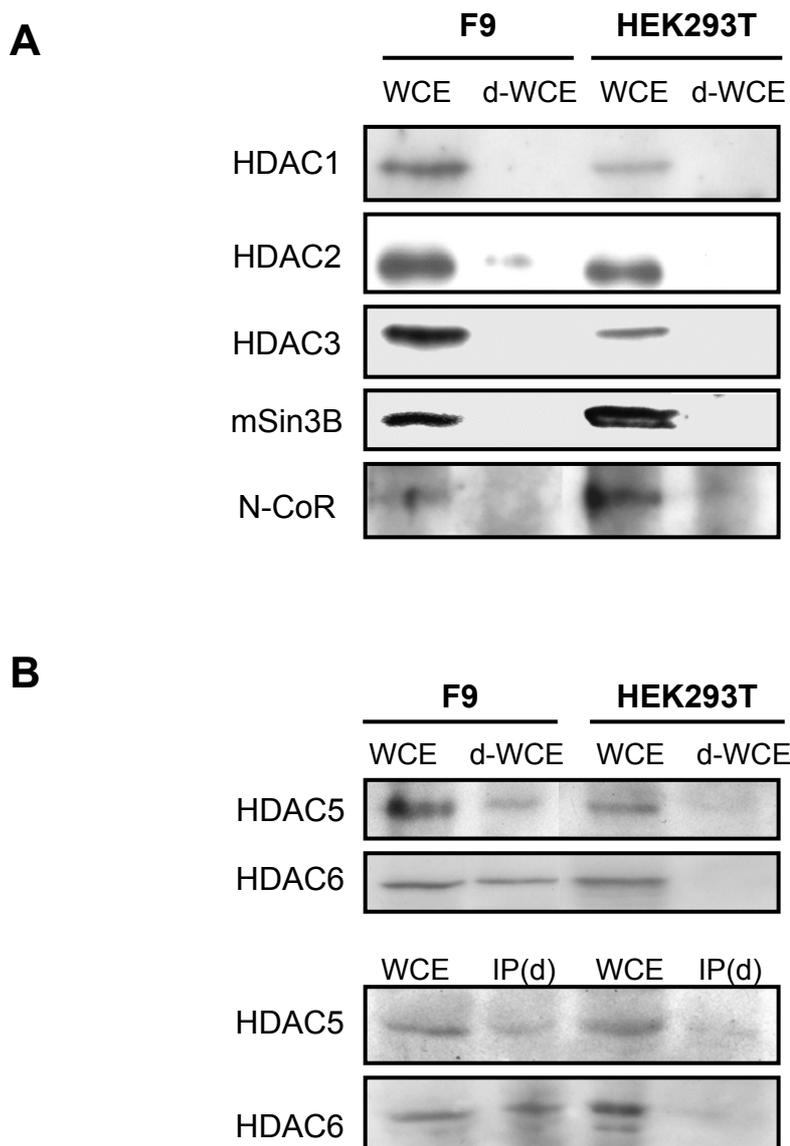


Figure 3.26 Existence and immunoprecipitation of HDACs 5 and 6 in F9 but not in HEK293T cell extracts depleted for N-CoR, mSin3, and HDACs 1-3. (A) Cell extracts were subjected to immunoprecipitation with a mixture of antibodies against N-CoR, mSin3, and HDACs 1-3 essentially as described for immunoprecipitation with individual antibodies. The depletion efficiency is shown by Western blot analysis of whole cell extracts before (WCE) and after (d-WCE) immunoprecipitation. (B) The depleted extracts were used for a second round of precipitation with antibodies against HDAC5 or 6. Immunoprecipitates were analyzed for HDAC activity (see Table 3.1) and subjected to Western blot analysis for the respective HDAC. The figure shows the amounts of HDAC5 and 6 in cell extracts (WCE and d-WCE) (upper panel) and immunoprecipitates from depleted extracts (IP (d)) compared to an equivalent amount of whole cell extract (WCE) from which the precipitate had been obtained (lower panel). Similar results were obtained in three independent experiments.

HDAC forms that are more sensitive to inhibition by VPA. In contrast, VPA significantly inhibits class I HDACs with IC₅₀-concentrations of 0.3 mM, 0.5 mM and 0.2 mM for HDAC1, 2 and 3, respectively (Table 3.1; Krämer, personal communication). Moreover, TSA inhibits the HDAC 5 and 6 precipitates with similar efficiency as the class I HDAC precipitates. Taken together, tested class II HDACs are approximately 5-10 folds less susceptible to inhibition by VPA than class I enzymes. Therefore VPA in contrast to TSA shows a substantial preference for inhibition of only a subset of HDACs, e.g. class I enzymes.

Table 3.1 Inhibition of anti-HDAC immunoprecipitates by VPA and TSA

IP	Source	IC ₅₀ VPA (mM)	IC ₅₀ TSA (nM)
HDAC2	F9	0.54 ± 0.22	0.64 ± 0.4
HDAC5	F9	1.8 ± 0.6	0.33 ± 0.04
HDAC6	F9	1.5 ± 0.8	0.33 ± 0.04
HDAC5	F9 depleted	2.8 ± 1.1	0.36 ± 0.17
HDAC6	F9 depleted	2.4 ± 1.0	0.29 ± 0.14

- a) IC₅₀ values were calculated as the concentrations that were required for 50% inhibition of HDAC activity. All reactions which showed a 20-85% inhibition were used for calculations according to the formula: IC₅₀ = Concentrations VPA or TSA / ((activity max / activity)-1).
- b) Values are means±SD from 4 to 8 individual determinations from at least 2 independent experiments.
- c) F9: F9 whole cell extract; F9 depleted: whole cell extract after immuno-depletion of HDAC1-3, N-CoR and mSin3B (Fig. 3.26 A).

3.3.5 Summary of this section

The results in this section clearly reveal that VPA inhibits HDACs *in vitro*, preferentially class I enzymes. In addition, VPA relieves HDAC-dependent transcriptional repression, and induces hyperacetylation of core histones in cultured cells and *in vivo*. Taken together, VPA is identified as a novel HDAC inhibitor showing isoenzyme selectivity (Göttlicher *et al.*, 2001). To the initial

question, HDAC inhibitory effect could be at least one of the mechanisms by which VPA activates PPAR δ -dependent transcription.

3.4 VPA selectively accelerates ubiquitin/proteasome-mediated HDAC2 degradation

It has been shown that some HDAC inhibitors like TSA up-regulate the mRNA levels of HDAC1-3 (Dangond and Gullans, 1998), suggesting an intriguing feedback loop. After defining VPA as a selective HDAC inhibitor, it was of interest to test whether and, if so, how VPA regulates the expression of HDACs.

3.4.1 VPA, but not TSA, selectively reduces HDAC2 protein levels

I first investigated HDAC expression upon VPA-treatment by Western blot analysis in several cell lines including F9, HEK293T and HT-29 cells. In all tested cell lines, VPA selectively down-regulated HDAC2 protein levels in a time- and dose-dependent manner. The substantial reduction of HDAC2 protein levels was detected between 8-24 hrs after VPA-exposure and persisted up to 48 hrs during exposure (Fig. 3.27 A). The delay of the reduction of HDAC2 protein suggests that the intermediate steps are required, e.g. regulation of the expression of genes responsible for reducing HDAC2 protein. In F9 cells, the dose sufficient for the reduction of HDAC2 protein is about 0.2-0.5 mM at which a clear down-regulation was observed (Fig. 3.27 A, left panels). Relatively moderate HDAC2 reduction was seen in HEK293T cells, and higher concentrations of VPA (e.g. 5 mM) caused more pronounced elimination of HDAC2 protein (Fig. 3.27 A, right panels; Krämer, personal communication). The protein levels of HDAC1 and 3, conversely, were not reduced and showed even a transient induction after VPA-exposure (Fig. 3.27 A, upper panels).

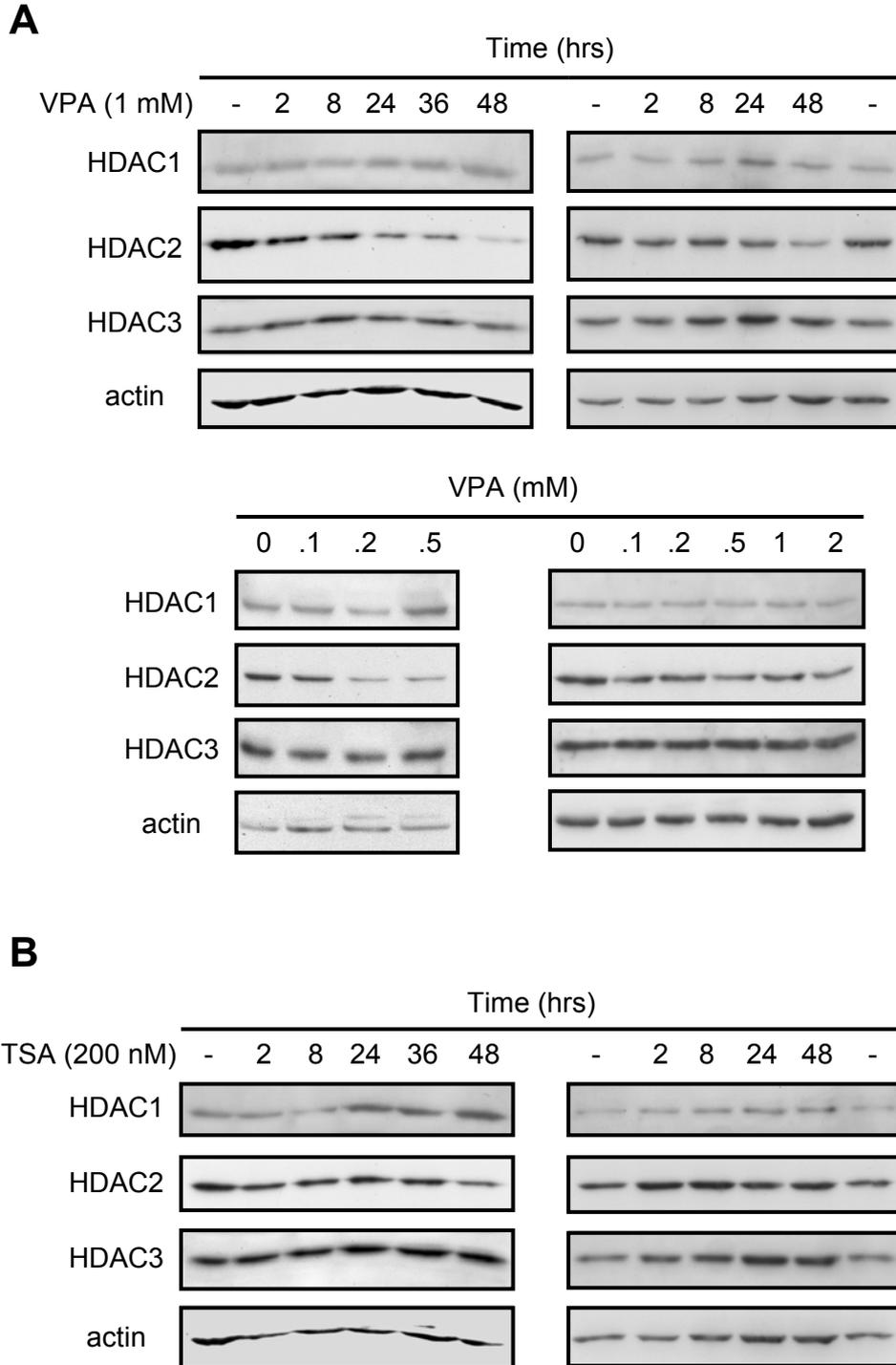


Figure 3.27 Reduction of HDAC2 protein levels by VPA but not by TSA. (A) After VPA-exposure, protein levels of HDAC2 and HDACs 1 or 3 for reference were determined by Western blot analysis. Time-dependency (1 mM VPA, upper panels) and dose-dependency (30 hrs exposure for F9 cells and 36 hrs for HEK293T cells, lower panel) are shown in F9 (left panels) and HEK293T (right panels) cells. (B) HDAC1-3 protein levels upon TSA-treatment were detected at indicated time points after exposure. Left panels: F9 cells, right panels: HEK293T cells. One representative is shown out of three (for F9 cells) or two (for HEK293T cells) independent experiments with similar results.

In contrast to VPA, the well-established HDAC inhibitor TSA, during up to 48 hrs of treatment did not down-regulate HDAC2 protein as well as HDAC1 and 3 in F9 and HEK293T cells (Fig. 3.27 B). Rather as described in literature, a slight induction of HDAC1 and 3 and a transient induction of HDAC2 (in HEK293T cells) were observed upon TSA-exposure (Dangond and Gullans, 1998). Moreover, other known HDAC inhibitors such as TSA, trapoxin and MS-275 did not cause HDAC2 down-regulation, suggesting reduction of HDAC2 protein is a specific effect of VPA (Fig. 3.23 B; Krämer, personal communication).

These results reveal another aspect of isoenzyme-selectivity of VPA compared to other HDAC inhibitors. Besides preferential inhibitory effect on the enzymatic activity of class I HDACs, VPA also selectively reduces HDAC2 protein levels.

3.4.2 VPA does not reduce the mRNA level of HDAC2

To elucidate at which level(s) VPA down-regulates HDAC2, the mRNA levels of HDAC2 were tested by Northern blot analysis after VPA-exposure. Under conditions that reduced HDAC2 protein levels, neither F9 and nor HEK293T cells showed a reduction of HDAC2 mRNA upon VPA-treatment (Fig. 3.28). In fact, HDAC2 mRNA was even up-regulated slightly upon VPA-exposure in HEK293T cells. This finding strongly suggests that HDAC2 down-regulation occurs at the translational or post-translational levels.

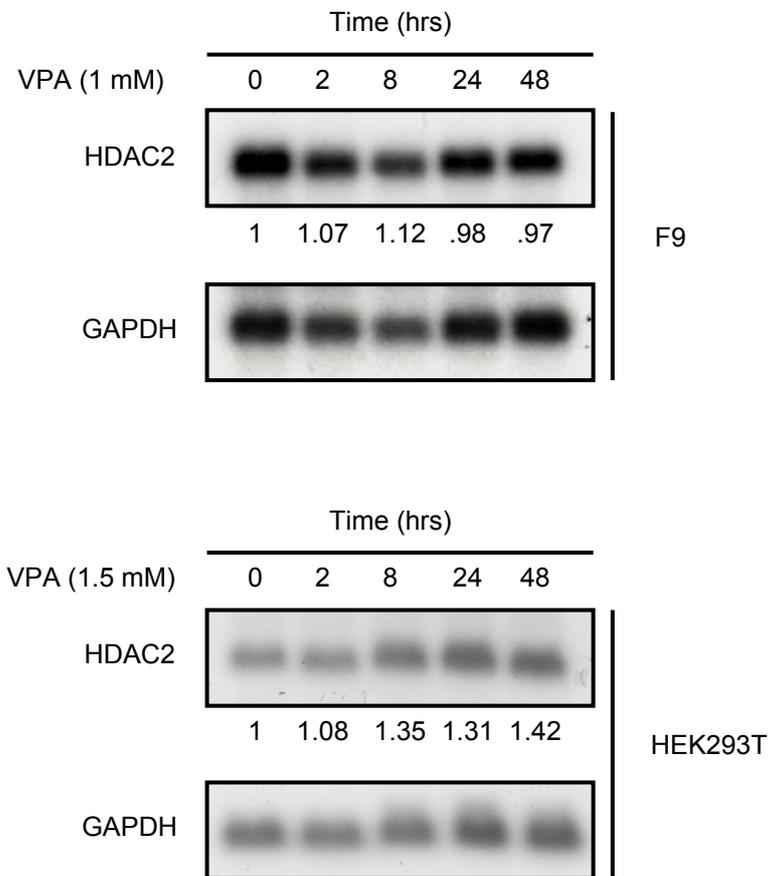


Figure 3.28 HDAC2 mRNA expression after VPA-treatment. F9 cells or HEK293T cells were treated for indicated times with 1 mM or 1.5 mM VPA, respectively. The levels of HDAC2 mRNA were determined by Northern blot analysis from 5 μ g poly(A)⁺ RNA. The same blots were re-hybridized with a GAPDH probe for loading control. The numbers below panels are relative amounts to untreated samples after normalization. Murine HDAC2 and GAPDH probes were also used for HEK293T cells based on the high identity of the sequences (>90%). Two independent experiments showed similar results.

3.4.3 VPA accelerates HDAC2 protein degradation

To evaluate whether VPA affects HDAC2 protein synthesis and/or protein degradation, metabolic pulse-labeling and pulse-chase experiments were carried out. Cells were metabolically pulse-labeled with ^{35}S -Met/Cys and chased with non-radioactive normal medium in the presence and absence of VPA. Subsequently HDAC2 or HDAC3 was immunoprecipitated from the cell lysates using specific antibodies. Newly synthesized ^{35}S -HDAC2 (or ^{35}S -HDAC3) in the immunoprecipitates was separated by SDS-PAGE and detected by autoradiography. In these experiments, the similar efficiencies of immunoprecipitations and the similar amounts of antibody used for immunoprecipitations were proven by Western blot analysis and Coomassie staining (data not shown). Both in F9 and in HEK293T cells, short-term (1 hr) pulse-labeling of HDAC2 with ^{35}S -Met/Cys indicated that the protein synthesis rate is not substantially changed in VPA-treated cells (24 hrs pretreatment and presence during labeling) as compared to untreated cells (Fig. 3.29).

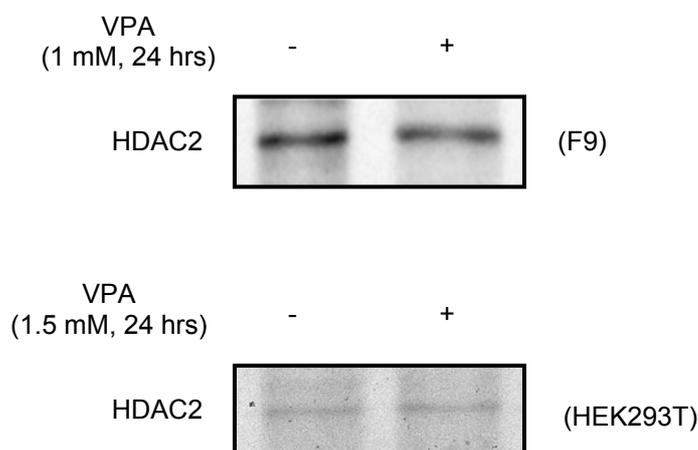


Figure 3.29 Synthesis rates of HDAC2 protein in the absence and presence of VPA. Protein synthesis rate of HDAC2 was analyzed by short-term (1 hr) pulse metabolic labeling with ^{35}S -Met/Cys. ^{35}S -labelled HDAC2 was detected by HDAC2 specific immunoprecipitation followed by SDS-PAGE separation and autoradiography. Pulse labeling was performed in F9 and HEK293T cells that had been left untreated (-) or exposed to VPA (doses indicated) for 24-hrs pretreatment and 1 hr during pulse labeling (+).

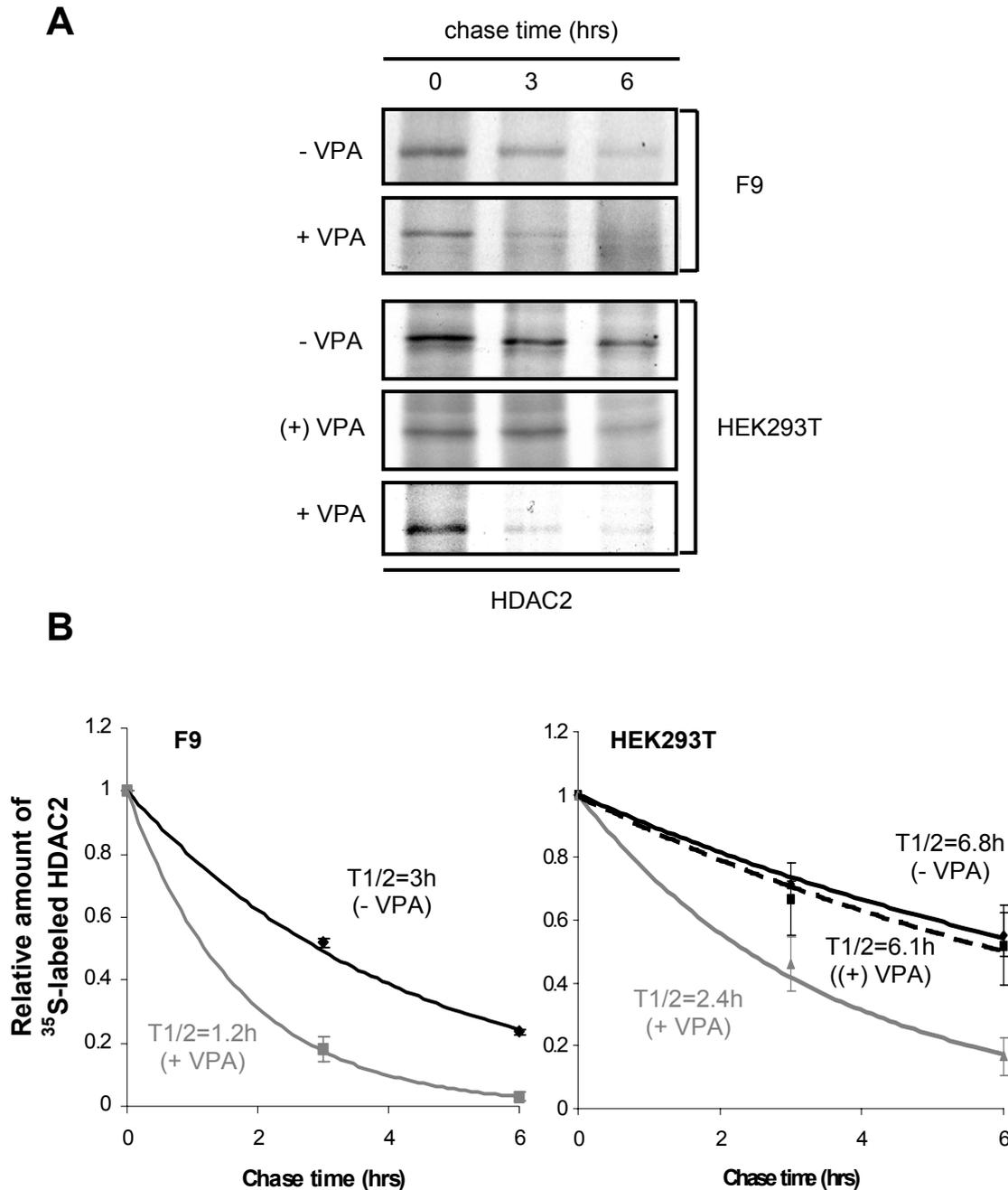


Figure 3.30 Increased HDAC2 protein degradation by VPA-treatment. (A) Protein degradation rate of HDAC2 was analyzed by a pulse-chase experiment. ³⁵S-labelled HDAC2 was detected by HDAC-2 specific immune precipitation followed by SDS-PAGE separation and autoradiography. Cells were left untreated (-VPA) or pretreated for 24 h with VPA (+VPA) (1 mM for F9 and 1.5 mM for HEK293T) and labeled with ³⁵S-Met/Cys for additional 3-hour in the absence or presence of VPA. After removal of ³⁵S-Met/Cys and addition of normal media the elimination of radiolabeled HDAC2 was followed over a period of 6 h (with or without VPA). A pulse chase analysis was also performed in HEK293T cells without VPA pretreatment and addition of VPA (indicated by "(+)VPA") only at the time when the chase period was started. (B) Graphs show the quantitative analysis and graphic representation of the data shown in (A) and in another independent experiment. The HDAC2 half-life times under different conditions were estimated from the graphs and indicated.

The half-life times (protein decay rates) of HDAC2 protein were determined by a pulse-chase analysis. The decay rates of HDAC2 were significantly increased in VPA-pretreated F9 and HEK293T cells (24 hrs pretreatment and presence during labeling and chase) (Fig. 3.30 A). In F9 cells the half-life time was reduced from 3 hrs (untreated control cells) to 1.2 hrs, and a decrease from 6 hrs to 2.3 hrs was found in HEK293T cells (Fig. 3.30 B). However, enhanced HDAC2 protein degradation was not observed when VPA was added only during chase period (Fig. 3.30). The last finding indicates that VPA-induced HDAC2 reduction is indirect rather than being direct.

In HEK293T cells, the degradation of HDAC3 was not affected upon VPA-pretreatment (Fig. 3.31). This observation is consistent with the non-reduced HDAC3 protein level upon VPA-exposure.

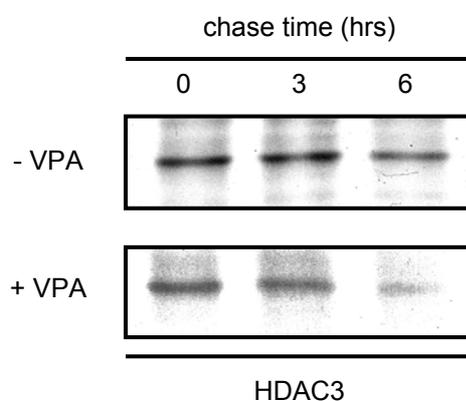


Figure 3.31 HDAC3 protein degradation in the absence and presence of VPA. The pulse chase analysis for HDAC3 was done in HEK 293T cells as that described for HDAC2 in Figure 3.30.

3.4.4 VPA elevates the ubiquitination of HDAC2 protein

Further analysis suggested that proteasome rather than proteases was involved in VPA-induced HDAC2 degradation (Krämer, personal communication). Since the 26S proteasome mainly recognizes poly-ubiquitinated proteins as the substrate, it was investigated whether would induce ubiquitination of HDAC2. An *in vivo* ubiquitination assay was carried out for this purpose by ectopic expression of His₆-tagged ubiquitin in HEK293T cells followed by a 36-hr VPA-treatment. The proteasome inhibitor MG-132 was added 4 hr prior to cell harvest to enhance accumulation of ubiquitinated proteins. With this pretreatment similar amounts of non-ubiquitinated HDAC2 were determined in each sample by Western blot analysis of whole cell lysate (Fig. 3.32, left panel). In precipitated His-tagged proteins, His₆-Ubi-transfected cells without VPA-treatment showed clear anti-HDAC2 immuno-reactive bands with the expected mobility of mono- and poly-ubiquitinated HDAC2. This observation suggested that HDAC2 is modified by ubiquitination under normal conditions. After 36 hrs VPA-treatment the degree of HDAC2 ubiquitination was substantially elevated (Fig. 3.32, upper right panel). An anti-HDAC2 immuno-reactive band about 60 KDa (similar to non-ubiquitinated HDAC2) was found in His₆-Ubi-transfected cells, which could represent a degraded ubiquitinated HDAC2 fragment or non-ubiquitinated HDAC2 in a precipitated protein complex containing His₆-ubiquitinated proteins (Fig. 3.32, upper right panel). On the other hand, VPA-treatment did not change the His₆-Ubiquitin expression and general ubiquitination of cellular proteins (Fig. 3.32, lower right panel). Enhanced ubiquitination of HDAC2 after VPA-treatment could be the prerequisite of VPA-induced HDAC2 protein degradation via the ubiquitin/proteasome pathway (Ciechanover *et al.*, 2000).

3.4.5 Summary of this section

This section shows that VPA, unlike other HDAC inhibitors, selectively down-regulates the HDAC2 protein levels. This reduction of HDAC2 is likely a consequence of VPA-induced HDAC2 protein degradation, probably mediated

by the ubiquitin/proteasome pathway, rather than regulation of mRNA expression or protein synthesis.

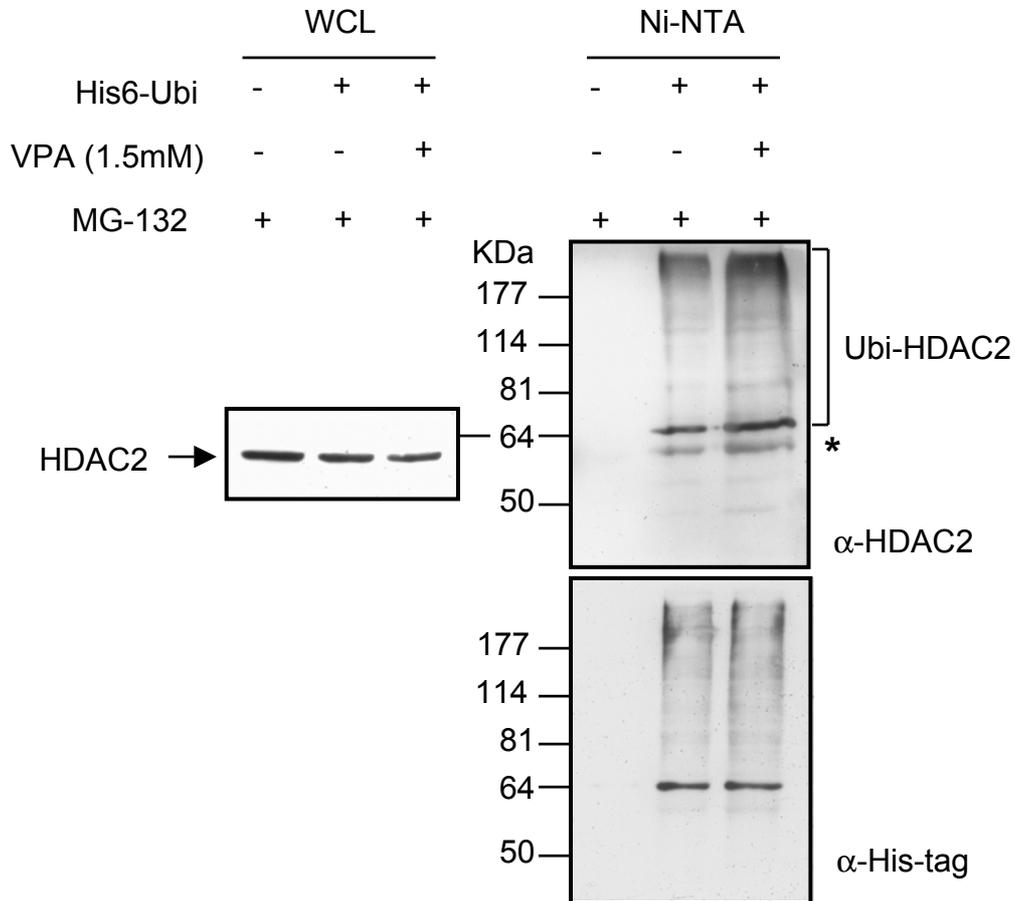


Figure 3.32 Ubiquitination of HDAC2 in the absence and presence of VPA. The presence of mono- and/ or poly-ubiquitinated HDAC2 upon VPA treatment of HEK293T cells was determined by ectopic expression of His₆-tagged ubiquitin followed by VPA-treatment for 36 hrs. The proteasome inhibitor MG-132 was added 4 h prior to analysis to enhance the accumulation of ubiquitinated proteins. The amounts of non-ubiquitinated HDAC2 were determined by Western blot analysis of whole cell extracts (left panel). Ubiquitinated proteins were precipitated with Ni²⁺-NTA-agarose and analyzed for the presence of ubiquitinated HDAC2 (upper-right panel) by Western blot analysis of precipitates. Anti-HDAC2 immunoreactive bands with the expected mobility of mono- or poly-ubiquitinated HDAC-2 are indicated. The asterisk (*) indicates a band of lower molecular weight that is discussed in the main text. Western blots of precipitates were also probed with an antibody directed against the poly-histidine tag (lower-right panel) to assure comparable expression of tagged ubiquitin and equal efficiency of incorporation of tagged ubiquitin into the general cellular pool of ubiquitinated proteins in control and VPA-treated cells.

4. Discussion

This thesis work presents three major findings. The first one was that VPA selectively inhibits class I HDACs, and in particular selectively induces HDAC2 protein degradation via the ubiquitin/proteasome pathway. The second finding was that HDAC2 expression is essential for the transformed phenotype of colorectal cancer cells. The last one was that HDAC2 expression could be regulated by PPAR δ .

4.1 VPA selectively targets class I HDAC, in particular HDAC2

4.1.1 VPA selectively inhibits class I HDAC activities

My data from *in vitro* HDAC assays (refer to 3.3) indicate that VPA, unlike TSA, preferentially acts on corepressor-associated HDACs and inhibits class I enzymes more efficiently than class II HDACs 5 and 6 at those concentrations that are also achieved in patient serum under therapy for epilepsy (0.2-1 mM). This observation suggests that VPA could block class I HDAC activities substantially whereas class II enzyme activities might be poorly influenced during epilepsy therapy. The isoenzyme-selectivity might be an explanation for the mild adverse effects of VPA even during long-term treatment. The exact significance of the selectivity is unclear due to the limited knowledge about the biological roles of distinct HDACs. Besides VPA, HDAC inhibitors trapoxin and the hybrid compound cyclic hydroxamic acid-containing peptide (CHAP) were also identified to possess isoenzyme-selectivity, which showed a much higher IC₅₀ for HDAC6 (Furumai *et al.*, 2001).

4.1.2 VPA selectively reduces HDAC2 protein level

As shown in section 3.4 and the data from Krämer *et al.* (personal communication), VPA selectively reduces protein levels of HDAC2 but not other class I isoenzymes in all tested cell lines (about 10 cell lines). In contrast, other HDAC inhibitors such as TSA and trapoxin show no down-

regulation of HDAC2 protein. This finding sends an important message that VPA not only preferentially inhibits the class I HDAC enzyme activities, but also selectively reduces HDAC2 protein among class I isoenzymes. From the results shown in this study, it is reasonable to suppose that at the concentrations around 0.5 mM which is realistic for clinical therapy, VPA is able to inhibit about half of the class I HDAC activity and even more severely block HDAC2 activity upon reducing its expression. To my knowledge, the discrimination of HDACs within the same class does not exist for any other HDAC inhibitor. This stringent selectivity could be beneficial for the clinical application of VPA in particular for those diseases, once identified, related to aberrant expression or recruitment of HDAC2, e.g. colorectal cancer as proposed below.

4.1.3 Mechanisms of VPA-induced HDAC2 down-regulation

The results in section 3.4 indicated that VPA-induced reduction of HDAC2 is likely due to an enhanced ubiquitin/proteasome-mediated protein degradation rather than transcriptional or translational regulation after VPA-exposure. The process of VPA-induced HDAC2 degradation appears to be an indirect effect, e.g. via inducing certain genes responsible for HDAC2 degradation rather than a direct effect (e.g. inducing degradation-favored conformational change of HDAC2), since the reduction of HDAC2 protein levels and HDAC2 protein degradation occur 16-24 hrs after VPA-treatment. It should be noted that VPA is a small diffusible molecule that could be distributed into cells very quickly.

Further experiments were carried out to search for the VPA-regulated molecules that are involved in HDAC2 degradation. Since VPA increases the ubiquitination of HDAC2 that could be a prerequisite of accelerated degradation, enzymes catalyzing ubiquitination were focused on. In a high throughput screening of VPA-regulated genes by the microarray technology, an E2 (Ubc) enzyme, Ubce8, was found to be substantially induced upon VPA-treatment. And overexpression of Ubce8 down-regulates HDAC2 protein levels (Golebiewski, personal communication). Moreover, RLIM, a newly identified RING finger domain E3 enzyme which ubiquitinates CLIM cofactors

(Ostendorff *et al.*, 2002), has been shown to catalyze the ubiquitination of HDAC2 *in vitro* in combination with Ubce8 as an E2 (Bach, personal communication). Therefore, candidate enzymes for HDAC2 degradation are proposed.

Ubiquitination of HDAC2 reveals a new pathway for regulation of HDAC2 expression. So far, several post-translational modifications have been identified for HDACs, including phosphorylation, SUMOylation and ubiquitination. Emerging evidence suggests that the covalent modifications play important roles for the activity, expression, cellular transportation and complex formation of HDACs (Galasinski *et al.*, 2002; David *et al.*, 2002; Kirsh *et al.*, 2002; Tsai and Seto, 2002). The interesting issue is whether targeted regulation of these modifications would affect the HDAC functions and consequently cellular processes, e.g. malignant transformation.

4.1.4 Anti-cancer effect of VPA

VPA has been shown to induce cellular differentiation and inhibit proliferation of human neuroblastoma cells, and to induce apoptosis in various human leukemia cell lines, despite the exact molecular mechanisms of the action are unknown (Cinatl *et al.*, 1997; Knupfer *et al.*, 1998; Andratschke *et al.*, 2001; Kawagoe *et al.*, 2002). Identification of VPA as a selective HDAC inhibitor carries a possible mechanistic explanation and expands its potential use for cancer therapy, since inhibition of HDACs is proposed to be of therapeutic value in many forms of cancer. At the concentrations of 0.2-1.0 mM that are achieved in patient serum during epilepsy therapy with a daily dose 20-30 mg/kg, VPA efficiently inhibits class I HDACs and induces histone hyperacetylation. And importantly, VPA induces differentiation and apoptosis of carcinoma cells, transformed hematopoietic progenitor cells and leukemic blasts from acute myeloid leukemia patients. Moreover, VPA significantly reduces tumor growth and metastasis in animal experiments. The restricted isoenzyme selectivity of VPA appears to suffice for induction of differentiation and apoptosis in transformed cells (Göttlicher *et al.*, 2001). Clinical trials are ongoing for both leukemia patients and non-hematologic cancer patients

(Göttlicher and Heinzel, personal communication). The advantage of VPA, compared to other HDAC inhibitors, is that it has been clinically used as an anti-epileptic drug for almost 30 years with well-documented pharmacological experience. (Göttlicher *et al.*, 2001). The teratogenic side effects have never seriously limited treatment of epilepsy with VPA. And in the expected treatment of cancer, the teratogenic adverse effects would only be relevant for female patients in the first trimester of pregnancy.

4.1.5 Correlation of HDAC inhibition and teratogenic activity of VPA-related compounds

Interestingly, for all VPA-related compounds tested so far, HDAC inhibition is tightly linked to teratogenicity but not anti-epileptic effect (Phiel *et al.*, 2001, Göttlicher *et al.*, 2001). Moreover, the well-established HDAC inhibitor TSA has strikingly similar teratogenic effects as VPA on developing *Xenopus* embryos (Phiel *et al.*, 2001). This observation suggests HDAC inhibition could be linked to teratogenicity. However, a mouse model argues against this proposition in which TSA did not cause apparent teratogenesis at the dose sufficient for inducing histone hyperacetylation (Nervi *et al.*, 2001). Further experiments are desired to figure out whether HDAC inhibition and teratogenicity are associated intrinsically.

4.2 Role of HDAC2 in colorectal cancer

4.2.1 Aberrant HDAC2 expression is essential for the transformed phenotype of HT-29 cells

Aberrant recruitment of HDAC activity has been linked to pathogenesis of certain leukemia and lymphoma (Krämer *et al.*, 2001; Marks *et al.*, 2001). HDAC inhibitors apparently induce differentiation and/or apoptosis of cancer cells derived from both hematologic and non-hematologic malignancies, suggesting aberrant HDAC activity could also be a cause of malignant transformation of non-hematopoietic cells. My study clearly reveals that

aberrantly high HDAC2 expression is essential for the transformed phenotype of colorectal cancer cells. This finding was mainly supported by observations from HT-29 colorectal cancer cell line that contains mutant form of the tumor suppressor gene *adenomatous polyposis coli (APC)* and undergoes apoptosis upon restoration of wild-type APC expression. Firstly, HDAC2 expression was found to be repressed by the presence of wild-type APC in HT-29 cells. Secondly and more importantly, Loss-of-function and gain-of-function analyses indicated that the elevated level of HDAC2 is required and sufficient for preventing apoptosis and maintaining transformed phenotype of colorectal cancer cells. These effects appear to be HDAC2-specific since RNAi technology was used for knock-down of HDAC2 in the loss-of-function analysis. This technology allows a specific reduction of HDAC2 without affecting other class I enzymes, e.g. HDAC1 and 3. Finally, most tested human colorectal carcinomas also display elevated HDAC2 protein levels compared to normal colonic tissues from the same patients, suggesting that HDAC2 is likely also to play a role during pathogenesis of colorectal cancer *in vivo*. To my knowledge, this finding exhibits the first evidence that aberrant HDAC expression could be important for pathogenesis of non-hematologic cancer.

Consistent with my observation that HDAC2 reduction substantially increases apoptosis in HT-29 cells, the apoptosis of colonic cancer cells has been shown to be associated with the hyperacetylation of histones (Wu *et al.*, 2001; Hinnebusch *et al.*, 2002). My study also shows that HDAC2 reduction does not affect cell cycle progression of HT-29 cells, arguing against previous proposition that HDAC2 might be involved in cell cycle regulation (Wang *et al.*, 2001). Noteworthy, it is difficult to assess different properties between HDAC1 and 2 since these two proteins frequently co-exist in the same corepressor complexes (Tsai and Seto, 2002). Remarkably, HDAC1 mainly contributes to enhancement of cell proliferation whereas does affect apoptosis as shown by knockout mice (Lagger *et al.*, 2002), suggesting a complementary role of HDAC1 and 2 in cell growth and neoplasia.

4.2.2 Questions to be answered: *in vivo* role, general oncogenic role of HDAC2 and HDAC2-regulated molecule(s)?

Although *in vitro* data substantially demonstrate a positive role of HDAC2 for the transformed phenotype of colonic cancer cells, the *in vivo* role of HDAC2 in colorectal tumorigenesis is still unclear. My results suggest that HDAC2 is likely implicated in pathogenesis of colorectal cancer *in vivo*, since elevated HDAC2 levels were detected in many samples of colorectal carcinomas. Whether HDAC2 plays a role in APC-dependent colorectal tumorigenesis *in vivo* could be eventually answered by crossing mice carrying one mutated APC allele (APC^{min}) (Su *et al.*, 1992) with HDAC2 knockout mice once they are generated.

Since mutations of APC and β -catenin widely occur in neoplasia (Morin, 1999), it is likely that HDAC2 could also be involved in tumors other than colorectal cancer. In fact, HDAC2 is overexpressed in lung and breast cancer as shown by immunohistochemistry (data not shown). Moreover, my data do not exclude that pathways other than APC/ β -catenin signaling could also cause aberrant HDAC2 expression. It is of interest to check whether HDAC2 plays a general oncogenic role in carcinogenesis, namely, whether HDAC2 overexpression itself is sufficient to trigger cell malignant transformation.

Another concern is to find out the transcription factors whose activities are aberrantly repressed due to HDAC2 overexpression and/or the genes regulated by HDAC2 that could be responsible for HDAC2-induced phenotype. The observation that HDAC2 mainly affects apoptosis in HT-29 cells carries the hint that certain genes controlling cell death might be regulated by HDAC2. It has been shown that HDAC inhibitors change the expression of p53 and Bcl members in certain experiments. Considering that HT-29 cells do not possess functional p53, anti-apoptosis factors Bcl-2 and Bcl-XL would be focused on in further investigations.

4.2.3 Perspectives of clinical relevance of HDAC2

Elucidating the role of HDAC2 in colorectal tumorigenesis might inspire the beneficial potentials for cancer therapy. Together with the other APC-regulated β -catenin/Tcf downstream molecules, e.g. c-myc (He *et al.*, 1998), cyclin D1 (Tetsu and McCormick, 1999) and PPAR δ (He *et al.*, 1999), HDAC2 could serve as a therapeutical target for the treatment of colorectal cancer. Besides targeted gene therapy which appears to be technically problematic (Mastrangelo *et al.*, 1996; Chang and He, 2001), employment of HDAC inhibitors could be a more desirable way for the therapy. Indeed, certain HDAC inhibitors, e.g. butyrates, have been shown to inhibit cell growth and to induce differentiation and apoptosis of colonic cancer cells (Heerdt *et al.*, 1994; Hague and Paraskeva, 1995; Avivi-Green *et al.*, 2002). Development of novel selective inhibitors, in particular HDAC2-specific inhibitor if possible, could be helpful for the treatment of cancer linked to aberrant HDAC2 expression.

4.3 PPAR δ

4.3.1 PPAR δ could be a regulator of HDAC2 expression

My work shows evidence that HDAC2 expression is positively regulated by PPAR δ either directly or indirectly in murine F9 and ES cells. Nevertheless, to ensure the role of PPAR δ in regulating HDAC2 expression, a more profound investigation should be carried out by using PPAR δ -deficient mice and human cell lines, e.g. PPAR δ -knockout HCT-116 cells (Park *et al.*, 2001).

Elucidation of the mechanism(s) by which PPAR δ regulates HDAC2 is also desired. Directly or indirectly transcriptional regulation could be tested by a reporter gene assay using the available sequence of murine HDAC2 promoter (Zeng *et al.*, 1998). However, lack of putative known PPAR δ binding sites argues against that the regulation is a direct effect.

In HT-29 colorectal cancer cells, PPAR δ may be a mediator of HDAC2 overexpression upon loss of wt APC, since PPAR δ is negatively regulated by APC. Alternative APC-regulated pathways should also be considered, e.g. c-myc-dependent signaling. Indeed, c-myc is a negative target gene of APC (He *et al.*, 1998). Most interestingly, there are several putative myc responsive elements located in murine HDAC2 promoter.

Endogenous HDAC2 protein could undergo ubiquitin/proteasome-mediated degradation (refer section 3.4). But it is unlikely that PPAR δ and APC regulate HDAC2 via this pathway since they do change HDAC2 expression at the mRNA levels.

Despite several proposed biological roles of PPAR δ , the downstream targets of PPAR δ that could contribute to functions of this receptor are largely unknown. The finding that PPAR δ could be a regulator of HDAC2 expression gives a hint that HDAC2 might play roles in certain PPAR δ -mediated processes, e.g. cell differentiation, apoptosis (Hatae *et al.*, 2001), embryo development and colorectal tumorigenesis. Indeed, this assumption seems to be reasonable since HDACs generally affects cellular processes such as proliferation, differentiation and apoptosis (Marks *et al.*, 2001). The functional relevance of PPAR δ and HDAC2 could be addressed by checking whether the phenotype could be alleviated by ectopic expression of HDAC2 in PPAR δ -deficient cells or mice, and by testing whether known PPAR δ functions are altered in HDAC2 knockout mice once they are available.

4.3.2 Mechanism of VPA-induced activation of PPAR δ -dependent transcription

Unliganded PPAR δ is a transcriptional repressor of basal transcription upon association with corepressor/HDAC complexes (Shi *et al.*, 2002). VPA could relieve PPAR δ -dependent transcriptional repression and allow at least partial activation probably in conjugation with low levels of endogenous PPAR δ ligands (Lampen *et al.*, 1999; Göttlicher *et al.*, 2001). Identification of VPA as

HDAC inhibitor reveals a new explanation for VPA-induced transactivation of PPAR δ . Since recruitment of corepressor-associated HDAC activities by unliganded PPAR δ is thought to be required for repression of PPAR δ -dependent gene expression, the HDAC inhibitory effect of VPA is likely the predominant mechanism by which VPA activates PPAR δ -dependent transcription.

Since HDAC2 gene expression might be induced by PPAR δ (refer to 4.3.1), one would expect that VPA could also up-regulate HDAC2 mRNA by activation of PPAR δ . However my experiments show that VPA does not affect HDAC2 mRNA levels in F9 cells, and it only slightly induces HDAC2 mRNA in HEK293T cells (refer to 3.4). It is possible that an endogenous ligand is persistent at a concentration that is sufficient for full activation of PPAR δ on HDAC2 gene expression. Consequently, VPA-treatment (or additional ligand) could have no impact on endogenous transactivation of PPAR δ , thus no effect on HDAC2 mRNA expression. This assumption is supported by the observations that only PPAR δ expression level but not the presence of additional ligand changes HDAC2 expression (refer to 3.1). Response of PPAR δ to VPA was detected only in the artificial settings of the reporter gene analysis, whereas under natural conditions it is difficult to test since no clear target gene of PPAR δ is known. Another possible explanation is that PPAR δ is only one of the transcription factors that regulate HDAC2 expression. VPA might differentially affect expression and the transcriptional activities of those regulators of HDAC2. Finally the overall effect on HDAC2 mRNA expression is very moderate after VPA-treatment. On the basis of current data, it is difficult to draw any firm conclusions. No matter how VPA regulates HDAC2 mRNA level, induced degradation and inhibition of HDAC activity appear to prevail over a putative induction of HDAC2 transcription (refer to 3.4 and 3.3).

4.4 Summary of the entire project

In summary, this thesis work reveals that: 1) VPA is a selective HDAC inhibitor that preferentially inhibits class I HDACs, and selectively induces HDAC2 protein degradation via the ubiquitin/proteasome pathway; 2) HDAC2 is essential for preventing apoptosis and maintaining the transformed phenotype of colorectal cancer cells; and 3) PPAR δ could regulate the expression of HDAC2. Based on the first and second findings and observations that VPA is able to inhibit the growth of a number of colorectal cancer cell lines (Martin, personal communication), VPA appears to be a suitable agent in the therapy of colon cancer patients.

5. References

- Afshar G, Murnane JP. (1999) Characterization of a human gene with sequence homology to *Saccharomyces cerevisiae* SIR2. *Gene* 234(1):161-8.
- Alland L, Muhle R, Hou H Jr, Potes J, Chin L, Schreiber-Agus N, DePinho RA. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* 387(6628): 49-55.
- Allfrey V, Faulkner RM, Mirsky AE. (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. . *Proc. Natl. Acad. Sci. USA.* 51: 786-94.
- Andratschke N, Grosu AL, Molls M, Nieder C. (2001) Perspectives in the treatment of malignant gliomas in adults. *Anticancer Res.* 21(5): 3541-50.
- Archer SY, Hodin RA. (1999) Histone acetylation and cancer. *Curr. Opin. Genet. Dev.* 9(2): 171-4.
- Ardinger HH, Atkin JF, Blackston RD, Elsas LJ, Clarren SK, Livingstone S, Flannery DB, Pellock JM, Harrod MJ, Lammer EJ, et al. (1988) Verification of the fetal valproate syndrome phenotype. *Am. J. Med. Genet.* 29(1): 171-85.
- Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K. (1989) Current Protocols in Molecular Biology. (New York: John Wiley and Sons, Inc.)
- Avivi-Green C, Polak-Charcon S, Madar Z, Schwartz B. (2002) Different molecular events account for butyrate-induced apoptosis in two human colon cancer cell lines. *J. Nutr.* 132(7): 1812-8.
- Ballas N, Battaglioli E, Atouf F, Andres ME, Chenoweth J, Anderson ME, Burger C, Moniwa M, Davie JR, Bowers WJ, Federoff HJ, Rose DW, Rosenfeld MG, Brehm P, Mandel G. (2001) Regulation of neuronal traits by a novel transcriptional complex. *Neuron* 31(3): 353-65.
- Barak Y, Liao D, He W, Ong ES, Nelson MC, Olefsky JM, Boland R, Evans RM. (2002) Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc. Natl. Acad. Sci. USA.* 99(1): 303-8.
- Bastie C, Holst D, Gaillard D, Jehl-Pietri C, Grimaldi PA. (1999) Expression of peroxisome proliferator-activated receptor PPARdelta promotes induction of PPARgamma and adipocyte differentiation in 3T3C2 fibroblasts. *J. Biol. Chem.* 274(31): 21920-5.

- Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W. (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382(6592): 638-42.
- Boffa LC, Vidali G, Mann RS, Allfrey VG. (1978) Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. *J. Biol. Chem.* 253(10): 3364-6.
- Bojic U, Elmazar MM, Hauck RS, Nau H. (1996) Further branching of valproate-related carboxylic acids reduces the teratogenic activity, but not the anticonvulsant effect. *Chem. Res. Toxicol.* 9(5): 866-70.
- Bosher JM, Labouesse M. (2000) RNA interference: genetic wand and genetic watchdog. *Nat. Cell Biol.* 2(2): E31-6.
- Boyes J, Byfield P, Nakatani Y, Ogryzko V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* 396(6711): 594-8.
- Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-54.
- Buggy JJ, Sideris ML, Mak P, Lorimer DD, McIntosh B, Clark JM. (2000) Cloning and characterization of a novel human histone deacetylase, HDAC8. *Biochem. J.* 350 Pt 1: 199-205.
- Chang LJ, He J. (2001) Retroviral vectors for gene therapy of AIDS and cancer. *Curr. Opin. Mol. Ther.* 3(5): 468-75.
- Ciechanover A, Orian A, Schwartz AL. (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22(5): 442-51.
- Cinatl J Jr, Cinatl J, Driever PH, Kotchetkov R, Pouckova P, Kornhuber B, Schwabe D. (1997) Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anticancer Drugs* 8(10): 958-63.
- Collins MD, Scott WJ, Miller SJ, Evans DA, Nau H. (1992) Murine teratology and pharmacokinetics of the enantiomers of sodium 2-ethylhexanoate. *Toxicol. Appl. Pharmacol.* 112(2): 257-65.

- Dangond F, Gullans SR. (1998) Differential expression of human histone deacetylase mRNAs in response to immune cell apoptosis induction by trichostatin A and butyrate. *Biochem. Biophys. Res. Commun.* 247(3): 833-7.
- David G, Neptune MA, DePinho RA. (2002) SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. *J. Biol. Chem.* 277(26): 23658-63.
- Desvergne B, Wahli W. (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.* 20(5): 649-88.
- DiLiberti JH, Farndon PA, Dennis NR, Curry CJ. (1984) The fetal valproate syndrome. *Am. J. Med. Genet.* 19(3): 473-81.
- Dressel U, Bailey PJ, Wang SC, Downes M, Evans RM, Muscat GE. (2001) A dynamic role for HDAC7 in MEF2-mediated muscle differentiation. *J. Biol. Chem.* 276(20): 17007-13.
- Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W. (1992) Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* 68(5): 879-87.
- Ehlers K, Sturje H, Merker HJ, Nau H. (1992) Valproic acid-induced spina bifida: a mouse model. *Teratology* 45(2): 145-54.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411(6836): 494-8.
- Elbashir SM, Harborth J, Weber K, Tuschl T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26(2): 199-213.
- Emiliani S, Fischle W, Van Lint C, Al-Abed Y, Verdin E. (1998) Characterization of a human RPD3 ortholog, HDAC3. *Proc. Natl. Acad. Sci. USA.* 95(6): 2795-800.
- Escher P, Wahli W. (2000) Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutat. Res.* 448(2): 121-38.
- Evans RM. (1988) The steroid and thyroid hormone receptor superfamily. *Science* 240(4854): 889-95.

- Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP. (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* 401(6749): 188-93.
- Fischle W, Emiliani S, Hendzel MJ, Nagase T, Nomura N, Voelter W, Verdin E. (1999) A new family of human histone deacetylases related to *Saccharomyces cerevisiae* HDA1p. *J. Biol. Chem.* 274(17): 11713-20.
- Forman BM, Chen J, Evans RM. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc. Natl. Acad. Sci. USA.* 94(9): 4312-7.
- Frye RA. (1999) Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem. Biophys. Res. Commun.* 260(1): 273-9.
- Frye RA. (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys. Res. Commun.* 273(2): 793-8.
- Furukawa Y, Kawakami T, Sudo K, Inazawa J, Matsumine A, Akiyama T, Nakamura Y. (1996) Isolation and mapping of a human gene (RPD3L1) that is homologous to RPD3, a transcription factor in *Saccharomyces cerevisiae*. *Cytogenet. Cell. Genet.* 73(1-2): 130-3.
- Furumai R, Komatsu Y, Nishino N, Khochbin S, Yoshida M, Horinouchi S. (2001) Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. *Proc. Natl. Acad. Sci. USA.* 98(1): 87-92.
- Galasinski SC, Resing KA, Goodrich JA, Ahn NG. (2002) Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions. *J. Biol. Chem.* 277(22): 19618-26.
- Gao L, Cueto MA, Asselbergs F, Atadja P. (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. *J. Biol. Chem.* 277(28): 25748-55.
- Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA. (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Mol. Cell Biol.* 18(12): 7185-91.

Glass CK, Rosenfeld MG. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* 14(2): 121-41.

Gottlicher M, Widmark E, Li Q, Gustafsson JA. (1992) Fatty acids activate a chimera of the clofibrilic acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. USA.* 89(10): 4653-7.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzl T. (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* 20(24): 6969-78.

Gray SG, Ekstrom TJ. (1998) Effects of cell density and trichostatin A on the expression of HDAC1 and p57Kip2 in Hep 3B cells. *Biochem. Biophys. Res. Commun.* 245(2): 423-7.

Gray SG, Ekstrom TJ. (2001) The human histone deacetylase family. *Exp. Cell Res.* 262(2): 75-83.

Green S, Kumar V, Theulaz I, Wahli W, Chambon P. (1988) The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. *EMBO J.* 7(10): 3037-44.

Grozinger CM, Hassig CA, Schreiber SL. (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl. Acad. Sci. USA.* 96(9): 4868-73.

Grozinger CM, Schreiber SL. (2000) Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proc. Natl. Acad. Sci. USA.* 97(14): 7835-40.

Gu W, Roeder RG. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90(4): 595-606.

Guardiola AR, Yao TP. (2002) Molecular cloning and characterization of a novel histone deacetylase HDAC10. *J. Biol. Chem.* 277(5): 3350-6.

Guenther MG, Barak O, Lazar MA. (2001) The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Mol. Cell Biol.* 21(18): 6091-101.

Gupta RA, Tan J, Krause WF, Geraci MW, Willson TM, Dey SK, DuBois RN. (2000) Prostacyclin-mediated activation of peroxisome proliferator-activated receptor delta in colorectal cancer. *Proc. Natl. Acad. Sci. USA.* 97(24): 13275-80.

- Hague A, Paraskeva C. (1995) The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. *Eur. J. Cancer Prev.* 4(5): 359-64.
- Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K. (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.* 114(Pt 24): 4557-65.
- Hatae T, Wada M, Yokoyama C, Shimonishi M, Tanabe T. (2001) Prostacyclin-dependent apoptosis mediated by PPAR delta. *J. Biol. Chem.* 276(49): 46260-7.
- He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. (1998) Identification of c-MYC as a target of the APC pathway. *Science* 281(5382): 1509-12.
- He TC, Chan TA, Vogelstein B, Kinzler KW. (1999) PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 99(3): 335-45.
- Heerdt BG, Houston MA, Augenlicht LH. (1994) Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res.* 54(12): 3288-93.
- Heinzel T, Lavinsky RM, Mullen TM, Soderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK, Rosenfeld MG. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* 387(6628): 43-8.
- Hershko A, Ciechanover A, Varshavsky A. (2000) Basic Medical Research Award. The ubiquitin system. *Nat. Med.* 6(10): 1073-81.
- Hinnebusch BF, Meng S, Wu JT, Archer SY, Hodin RA. (2002) The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J. Nutr.* 132(5): 1012-7.
- Horie S, Suga T. (1985) Enhancement of peroxisomal beta-oxidation in the liver of rats and mice treated with valproic acid. *Biochem. Pharmacol.* 34(9): 1357-62.
- Hu E, Kim JB, Sarraf P, Spiegelman BM. (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* 274: 2100-3.

Hu E, Chen Z, Fredrickson T, Zhu Y, Kirkpatrick R, Zhang GF, Johanson K, Sung CM, Liu R, Winkler J. (2000) Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. *J. Biol. Chem.* 275(20): 15254-64.

Huang EY, Zhang J, Miska EA, Guenther MG, Kouzarides T, Lazar MA. (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev.* 14(1): 45-54.

Huot C, Gauthier M, Lebel M, Larbrisseau A. (1987) Congenital malformations associated with maternal use of valproic acid. *Can. J. Neurol. Sci.* 14(3): 290-3.

Hutvagner G, Zamore PD. (2002) RNAi: nature abhors a double-strand. *Curr. Opin. Genet. Dev.* 12(2): 225-32.

Issemann I, Green S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347(6294): 645-50.

Johannessen CU. (2000) Mechanisms of action of valproate: a commentary. *Neurochem. Int.* 37(2-3): 103-10.

Juge-Aubry CE, Hammar E, Siegrist-Kaiser C, Pernin A, Takeshita A, Chin WW, Burger AG, Meier CA. (1999) Regulation of the transcriptional activity of the peroxisome proliferator-activated receptor alpha by phosphorylation of a ligand-independent trans-activating domain. *J. Biol. Chem.* 274(15): 10505-10.

Kao HY, Downes M, Ordentlich P, Evans RM. (2000) Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. *Genes Dev.* 14(1): 55-66.

Kao HY, Lee CH, Komarov A, Han CC, Evans RM. (2002) Isolation and characterization of mammalian HDAC10, a novel histone deacetylase. *J. Biol. Chem.* 277(1): 187-93.

Kawagoe R, Kawagoe H, Sano K. (2002) Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. *Leuk. Res.* 26(5): 495-502.

Kersten S, Desvergne B, Wahli W. (2000) Roles of PPARs in health and disease. *Nature* 405(6785): 421-4.

Kim YB, Lee KH, Sugita K, Yoshida M, Horinouchi S. (1999) Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. *Oncogene* 18(15): 2461-70.

Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW. (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat. Med.* 7(4): 437-43.

Kinzler KW, Vogelstein B. (1996) Lessons from hereditary colorectal cancer. *Cell* 87(2): 159-70.

Kirsh O, Seeler JS, Pichler A, Gast A, Muller S, Miska E, Mathieu M, Harel-Bellan A, Kouzarides T, Melchior F, Dejean A. (2002) The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. *EMBO J.* 21(11): 2682-91.

Kliwer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, Evans RM. (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA.* 91(15): 7355-9.

Kliwer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc. Natl. Acad. Sci. USA.* 94(9): 4318-23.

Knupfer MM, Hernaiz-Driever P, Poppenborg H, Wolff JE, Cinatl J. (1998) Valproic acid inhibits proliferation and changes expression of CD44 and CD56 of malignant glioma cells in vitro. *Anticancer Res.* 18(5A): 3585-9.

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC^{-/-} colon carcinoma. *Science* 275(5307): 1784-7.

Kramer OH, Gottlicher M, Heinzl T. (2001) Histone deacetylase as a therapeutic target. *Trends Endocrinol. Metab.* 12(7): 294-300.

Krogsdam AM, Nielsen CA, Neve S, Holst D, Helledie T, Thomsen B, Bendixen C, Mandrup S, Kristiansen K. (2002) Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-activated receptor delta-mediated transactivation. *Biochem. J.* 363(Pt 1): 157-65.

Kwon HJ, Kim MS, Kim MJ, Nakajima H, Kim KW. (2002) Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. *Int. J. Cancer* 97(3): 290-6.

Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-5.

Lagger G, O'Carroll D, Rembold M, Khier H, Tischler J, Weitzer G, Schuettengruber B, Hauser C, Brunmeir R, Jenuwein T, Seiser C. (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *EMBO J.* 21(11): 2672-81.

Laherty CD, Yang WM, Sun JM, Davie JR, Seto E, Eisenman RN. (1997) Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* 89(3): 349-56.

Lampen A, Siehler S, Ellerbeck U, Gottlicher M, Nau H. (1999) New molecular bioassays for the estimation of the teratogenic potency of valproic acid derivatives in vitro: activation of the peroxisomal proliferator-activated receptor (PPARdelta). *Toxicol. Appl. Pharmacol.* 160(3): 238-49.

Lampen A, Carlberg C, Nau H. (2001) Peroxisome proliferator-activated receptor delta is a specific sensor for teratogenic valproic acid derivatives. *Eur. J. Pharmacol.* 431(1): 25-33.

Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG, Kouzarides T. (2002) Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J.* 21(10): 2383-96.

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* 270(22): 12953-6.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 272(6): 3406-10.

Lehrach H, Diamond D, Wozney JM, Boedtker H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* 16(21): 4743-51.

Lehtonen E, Laasonen A, Tienari J. (1989) Teratocarcinoma stem cells as a model for differentiation in the mouse embryo. *Int. J. Dev. Biol.* 33(1): 105-15.

Leibowitz MD, Fievet C, Hennuyer N, Peinado-Onsurbe J, Duez H, Bergera J, Cullinan CA, Sparrow CP, Baffic J, Berger GD, Santini C, Marquis RW, Tolman RL, Smith RG, Moller DE,

Auwerx J. (2000) Activation of PPARdelta alters lipid metabolism in db/db mice. *FEBS Lett.* 473(3): 333-6.

Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK. (1999) Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev.* 13(12): 1561-74.

Loscher W. (1999) Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. *Prog. Neurobiol.* 58(1): 31-59.

Lu J, McKinsey TA, Zhang CL, Olson EN. (2000) Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol. Cell* 6(2): 233-44.

Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 107(2): 137-48.

Lutterbach B, Westendorf JJ, Linggi B, Patten A, Moniwa M, Davie JR, Huynh KD, Bardwell VJ, Lavinsky RM, Rosenfeld MG, Glass C, Seto E, Hiebert SW. (1998) ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol. Cell Biol.* 18(12): 7176-84.

Magge SS, Guardiola-Diaz HM. (2002) Characterization of the mouse peroxisome proliferator-activated receptor delta gene. *Biochem. Biophys. Res. Commun.* 290(1): 230-5.

Mal A, Sturniolo M, Schiltz RL, Ghosh MK, Harter ML. (2001) A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. *EMBO J.* 20(7): 1739-53.

Mangelsdorf DJ, Evans RM. (1995) The RXR heterodimers and orphan receptors. *Cell* 83(6): 841-50.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell* 83(6): 835-9.

Marks PA, Richon VM, Rifkind RA. (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J. Natl. Cancer Inst.* 92(15): 1210-6.

Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. (2001) Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* 1(3): 194-202.

Martinez-Frias ML. (1991) Valproic acid and spina bifida. *Lancet* 338(8760): 196-7.

Mastrangelo MJ, Berd D, Nathan FE, Lattime EC. (1996) Gene therapy for human cancer: an essay for clinicians. *Semin. Oncol.* 23(1): 4-21.

Matsumoto H, Erickson RH, Gum JR, Yoshioka M, Gum E, Kim Y. (1990) Biosynthesis of alkaline phosphatase during differentiation of the human colon cancer cell line Caco-2. *Gastroenterology* 98: 1199-1207.

McCampbell A, Taye AA, Whitty L, Penney E, Steffan JS, Fischbeck KH. (2001) Histone deacetylase inhibitors reduce polyglutamine toxicity. *Proc. Natl. Acad. Sci. USA.* 98(26): 15179-84.

McKinsey TA, Zhang CL, Lu J, Olson EN. (2000) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* 408(6808): 106-11.

Michalik L, Desvergne B, Tan NS, Basu-Modak S, Escher P, Rieusset J, Peters JM, Kaya G, Gonzalez FJ, Zakany J, Metzger D, Chambon P, Duboule D, Wahli W. (2001) Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice. *J. Cell Biol.* 154(4): 799-814.

Minucci S, Maccarana M, Cioce M, De Luca P, Gelmetti V, Segalla S, Di Croce L, Giavara S, Matteucci C, Gobbi A, Bianchini A, Colombo E, Schiavoni I, Badaracco G, Hu X, Lazar MA, Landsberger N, Nervi C, Pelicci PG. (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. *Mol. Cell* 5(5): 811-20.

Minucci S, Nervi C, Lo Coco F, Pelicci PG. (2001) Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias? *Oncogene* 20(24): 3110-5.

Miska EA, Karlsson C, Langley E, Nielsen SJ, Pines J, Kouzarides T. (1999) HDAC4 deacetylase associates with and represses the MEF2 transcription factor. *EMBO J.* 18(18): 5099-107.

Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destree O, Clevers H. (1996) XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* 86(3): 391-9.

Morin PJ, Vogelstein B, Kinzler KW. (1996) Apoptosis and APC in colorectal tumorigenesis. *Proc. Natl. Acad. Sci. USA.* 93(15): 7950-4.

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275(5307): 1787-90.

Morin PJ. (1999) beta-catenin signaling and cancer. *Bioessays* 21(12): 1021-30.

Muller-Oerlinghausen B, Berghofer A, Bauer M. (2002) Bipolar disorder. *Lancet* 359(9302): 241-7.

Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. (1995) Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc. Natl. Acad. Sci. USA.* 92(7): 3046-50.

Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 89(3): 373-80.

Nau H, Loscher W. (1986) Pharmacologic evaluation of various metabolites and analogs of valproic acid: teratogenic potencies in mice. *Fundam. Appl. Toxicol.* 6(4): 669-76.

Nau H, Hauck RS, Ehlers K. (1991) Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. *Pharmacol Toxicol.* 69(5): 310-21.

Nervi C, Borello U, Fazi F, Buffa V, Pelicci PG, Cossu G. (2001) Inhibition of histone deacetylase activity by trichostatin A modulates gene expression during mouse embryogenesis without apparent toxicity. *Cancer Res.* 61(4): 1247-9.

Nuclear Receptors Nomenclature Committee. (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97: 161-3.

Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. (2001) A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc. Natl. Acad. Sci. USA.* 98(9): 5306-11.

Ostendorff HP, Peirano RI, Peters MA, Schluter A, Bossenz M, Scheffner M, Bach I. (2002) Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* 416(6876): 99-103.

Park BH, Vogelstein B, Kinzler KW. (2001) Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells. *Proc. Natl. Acad. Sci. USA.* 98(5): 2598-603.

Parsons PG, Hansen C, Fairlie DP, West ML, Danoy PA, Sturm RA, Dunn IS, Pedley J, Ablett EM. (1997) Tumor selectivity and transcriptional activation by azelaic bishydroxamic acid in human melanocytic cells. *Biochem. Pharmacol.* 53(11): 1719-24.

Pazin MJ, Kadonaga JT. (1997) What's up and down with histone deacetylation and transcription? *Cell* 89(3): 325-8.

Peters JM, Lee SS, Li W, Ward JM, Gavrillova O, Everett C, Reitman ML, Hudson LD, Gonzalez FJ. (2000) Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). *Mol. Cell Biol.* 20(14): 5119-28.

Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J. Biol. Chem.* 276(39): 36734-41.

Pickart CM. (2001) Ubiquitin enters the new millennium. *Mol. Cell* 8(3): 499-504.

Pogo BG, Allfrey VG, Mirsky AE. (1966) RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. *Proc. Natl. Acad. Sci. USA.* 55(4): 805-12.

Polakis P. (2000) Wnt signaling and cancer. *Genes Dev.* 14(15): 1837-51.

Ponchaut S, Draye JP, Veitch K, Van Hoof F. (1991) Influence of chronic administration of valproate on ultrastructure and enzyme content of peroxisomes in rat liver and kidney. Oxidation of valproate by liver peroxisomes. *Biochem. Pharmacol.* 41(10): 1419-28.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. (1992) APC mutations occur early during colorectal tumorigenesis. *Nature* 359(6392): 235-7.

Puri PL, Iezzi S, Stiegler P, Chen TT, Schiltz RL, Muscat GE, Giordano A, Kedes L, Wang JY, Sartorelli V. (2001) Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. *Mol. Cell* 8(4): 885-97.

Qiu L, Kelso MJ, Hansen C, West ML, Fairlie DP, Parsons PG. (1999) Anti-tumour activity in vitro and in vivo of selective differentiating agents containing hydroxamate. *Br. J. Cancer* 80(8): 1252-8.

Rastinejad F, Perlmann T, Evans RM, Sigler PB. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature* 375(6528): 203-11.

Reese DH, Fiorentino GJ, Claflin AJ, Malinin TI, Politano VA. (1981) Rapid induction of alkaline phosphatase activity by retinoic acid. *Biochem. Biophys. Res. Commun.* 102(1): 315-21.

Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular Cloning. A Laboratory Manual (Second edition). *Cold Spring Harbor Laboratory Press*.

Schmidt A, Endo N, Rutledge SJ, Vogel R, Shinar D, Rodan GA. (1992) Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* 6: 1634-1641.

Schwabe JW, Fairall L, Chapman L, Finch JT, Dutnall RN, Rhodes D. (1993) The cocrystal structures of two zinc-stabilized DNA-binding domains illustrate different ways of achieving sequence-specific DNA recognition. *Cold Spring Harb. Symp. Quant. Biol.* 58:141-7.

Shao D, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, Lazar MA. (1998) Interdomain communication regulating ligand binding by PPAR-gamma. *Nature* 396(6709): 377-80.

Sher T, Yi HF, McBride OW, Gonzalez FJ. (1993) cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry* 32(21): 5598-604.

Shi Y, Hon M, Evans RM. (2002) The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. *Proc. Natl. Acad. Sci. USA.* 99(5): 2613-8.

Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, Apostol BL, Kazantsev A, Schmidt E, Zhu YZ, Greenwald M, Kurokawa R, Housman DE, Jackson GR, Marsh JL, Thompson LM.

(2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature* 413(6857): 739-43.

Struhl K. (1998) Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* 12(5): 599-606.

Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. (1992) Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 256(5057): 668-70.

Tan NS, Michalik L, Noy N, Yasmin R, Pacot C, Heim M, Fluhmann B, Desvergne B, Wahli W. (2001) Critical roles of PPAR beta/delta in keratinocyte response to inflammation. *Genes Dev.* 15(24): 3263-77.

Taunton J, Hassig CA, Schreiber SL. (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* 272(5260): 408-11.

Tetsu O, McCormick F. (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 398(6726): 422-6.

Tong JJ, Liu J, Bertos NR, Yang XJ. (2002) Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. *Nucleic Acids Res.* 30(5): 1114-23.

Tsai SC, Seto E. (2002) Regulation of histone deacetylase-2 by protein kinase CK2. *J. Biol. Chem.* [Jun 24 epub. ahead of print]

Van den Wyngaert I, de Vries W, Kremer A, Neefs J, Verhasselt P, Luyten WH, Kass SU. (2000) Cloning and characterization of human histone deacetylase 8. *FEBS Lett.* 478(1-2): 77-83.

Van Lint C, Emiliani S, Verdin E. (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr.* 5(4-5): 245-53.

Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107(2): 149-59.

Verdel A, Curtet S, Brocard MP, Rousseaux S, Lemerrier C, Yoshida M, Khochbin S. (2000) Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. *Curr. Biol.* 10(12): 747-9.

- Wang AH, Bertos NR, Vezmar M, Pelletier N, Crosato M, Heng HH, Th'ng J, Han J, Yang XJ. (1999) HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol. Cell Biol.* 19(11): 7816-27.
- Wang AH, Kruhlak MJ, Wu J, Bertos NR, Vezmar M, Posner BI, Bazett-Jones DP, Yang XJ. (2000) Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. *Mol. Cell Biol.* 20(18): 6904-12.
- Wang C, Fu M, Mani S, Wadler S, Senderowicz AM, Pestell RG. (2001) Histone acetylation and the cell-cycle in cancer. *Front. Biosci.* 6: D610-29.
- Warrell RP Jr, He LZ, Richon V, Calleja E, Pandolfi PP. (1998) Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* 90(21): 1621-5.
- Wen YD, Perissi V, Staszewski LM, Yang WM, Kroner A, Glass CK, Rosenfeld MG, Seto E. (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc. Natl. Acad. Sci. USA.* 97(13): 7202-7.
- Werling U, Siehler S, Litfin M, Nau H, Gottlicher M. (2001) Induction of differentiation in F9 cells and activation of peroxisome proliferator-activated receptor delta by valproic acid and its teratogenic derivatives. *Mol. Pharmacol.* 59(5): 1269-76.
- Williams RS, Cheng L, Mudge AW, Harwood AJ. (2002) A common mechanism of action for three mood-stabilizing drugs. *Nature* 417(6886): 292-5.
- Wu H, Olson EN. (2002) Activation of the MEF2 transcription factor in skeletal muscles from myotonic mice. *J. Clin. Invest.* 109(10): 1327-33.
- Wu JT, Archer SY, Hinnebusch B, Meng S, Hodin RA. (2001) Transient vs. prolonged histone hyperacetylation: effects on colon cancer cell growth, differentiation, and apoptosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280(3): G482-90.
- Wyllie AH. (1995) The genetic regulation of apoptosis. *Curr. Opin. Genet. Dev.* 5(1): 97-104.
- Xing G, Zhang L, Zhang L, Heynen T, Yoshikawa T, Smith M, Weiss S, Detera-Wadleigh S. (1995) Rat PPAR delta contains a CGG triplet repeat and is prominently expressed in the thalamic nuclei. *Biochem. Biophys. Res. Commun.* 217(3): 1015-25.

Xu L, Glass CK, Rosenfeld MG. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.* 9(2): 140-7.

Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA, Milburn MV. (1999) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* 3(3): 397-403.

Xu HE, Lambert MH, Montana VG, Plunket KD, Moore LB, Collins JL, Oplinger JA, Kliewer SA, Gampe RT Jr, McKee DD, Moore JT, Willson TM. (2001) Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA.* 98(24): 13919-24.

Yang WM, Inouye C, Zeng Y, Bearss D, Seto E. (1996) Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc. Natl. Acad. Sci. USA.* 93(23): 12845-50.

Yang WM, Yao YL, Sun JM, Davie JR, Seto E. (1997) Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. *J. Biol. Chem.* 272(44): 28001-7.

Yao YL, Yang WM, Seto E. (2001) Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell Biol.* 21(17): 5979-91.

Yoshida M, Kijima M, Akita M, Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* 265(28): 17174-9.

Zeng Y, Tang CM, Yao YL, Yang WM, Seto E. (1998) Cloning and characterization of the mouse histone deacetylase-2 gene. *J. Biol. Chem.* 273(44): 28921-30.

Zhou Q, Melkounian ZK, Lucktong A, Moniwa M, Davie JR, Strobl JS. (2000) Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *J. Biol. Chem.* 275(45): 35256-63.

Zhou X, Marks PA, Rifkind RA, Richon VM. (2001) Cloning and characterization of a histone deacetylase, HDAC9. *Proc. Natl. Acad. Sci. USA.* 98(19): 10572-7.