UNIVERSITÉ DE STRASBOURG

THÈSE présentée par :

Marie-Elodie SPAETY

soutenue le : **14 septembre 2016**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

Implication des régulations épigénétiques dans la réponse aux chimiothérapies dans les cancers gastriques – Perspectives thérapeutiques

THÈSE dirigée par :
  Mr GAIDDON Christian   Directeur de Recherche, Université de Strasbourg
  Mr PFEFFER Sébastien   Directeur de Recherche, Université de Strasbourg

RAPPORTEURS :
  Mr SAVA Gianni   PR PUPH, Université de Trieste
  Mr JUNG Manfred   PR PUPH, Université de Freiburg

AUTRES MEMBRES DU JURY :
  Mme MASSON Murielle   Chargé de recherche, Université de Strasbourg
  Mr BARBRY Pascal   Directeur de recherche, IPMC Valbonne

TABLE OF CONTENTS

ABBREVIATIONS

5-FU	5-Fluorouracil
ACRG	Asian Cancer Research Group
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AGC	Advanced Gastric Cancer
Ago	Argonaute
AML	Acute Myeloid Leukemia
AMPK	AMP-activated protein kinase
APAF1	Apoptotic Peptidase Activating Factor 1
AQP2/9	Aquaporin
ARF	ADP ribosylation factor
ARF-BP1	ADP ribosylation factor-Binding Protein 1
ARID1A	AT-Rich Interaction Domain 1A
ASPP	Apoptosis-Stimulating Protein of p53
ATF4/6	Activating Transcription Factor
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine TriPhosphate
ATP7A/B	ATPase copper transporting alpha
ATR	Ataxia telangiectasia and Rad3-related protein
BabA	Blood group Ag- binding adhesin
BAX	BCL2-associated X protein
BBC3	Bcl-2-Binding Component 3
BCL-6/2	B-Cell Lymphoma
Bcl-XL	B-Cell Lymphoma-extra Large
BMP	Bone marrow protein
BRCA1	Breast Cancer 1
BTB/POZ	Broad complex, Tramtrack and bric a brac / Poxvirus and Zing finger
Bub1	Budding Uninhibited by Benzimidazoles 1
C-EBP	CCAAT-enhancer-binding protein
CagA	Cytotoxin-associated gene A
Cag PAI	Cag Pathogenicity Island
CAMK	Calcium-calmodulin dependent protein Kinase
CAT	Catalase

CBP	CREB-Binding Protein
CBS	Cystathionine beta synthase
CCDN2	Cyclin-D2
CCR4	C-C chemokine receptor type 4
CD71	Transferrin Receptor
CDH1	Cadherin-1
CDK	Cyclin Dependent Kinase
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CHAC1	Human Cation transport regulator like protein 1
CHD	Chromodomain and Helicase Domain
Chk1	Checkpoint 1
CHOP	CCAAT-Enhancer-Binding protein Homologous Protein
CIN	Chromosomal Instability tumors
CK1	Casein Kinase 1
CLL	Chronic Lymphocytic Leukemia
CLL	Chronic Lymphocytic Leukemia
CMR1	Changed Mutation Rate 1
CtBP	C-terminal-binding protein
CTCL	Cutaneous T-Cell Lymphoma
CTD	Carboxi-Terminal domain
CTNNA1	Catenin Alpha-1
CTR1	Copper Transporter 1
CTH	Cystathionine gamma-lyase
DAC	5-Aza-2'-deoxycytidine
DACH	DiaminoCycloHexane ligand
DBD	DNA binding domain
DCP1/2	Decapping Protein
DGC	Distal Gastric Cancer
DGRC8	DiGeorge syndrome critical Region 8
DMSO	Dimethyl sulfoxide
DNA	DeoxyriboNucleic Acid
DNMT1/3	DNA Methyltransferase
DR5	Death Receptor 5
dsRNA	double-strand RNA

EBNA3C	Epstein-Barr Virus Nuclear antigen 6
EBV	Epstein Barr Virus
ECM	ExtraCellular Matrix
EGC	Early Gastric Cancer
EGFR	Epidermal Growth Factor Receptor
EIF2a	Eukaryotic Translation Initiation Factor 2a
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
ERAD	ER-Associated Protein Degradation
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERCC1	Excision Repair Cross-Complementation group 1
ERK	Extracellular signal-Regulated Kinases
Ero1	Endoplasmic Reticulum Oxidoreductin 1
ES cells	Embryonic Stem cells
EXO1	Exonuclease 1
EXP5	Exportin 5
EZH2	Enhancer of zeste homolog 2
FAK	Focal Adhesion Kinase
FDA	Food and Drug Administration
FIGC	Familial Intestinal Gastric Cancer
GADD45	45kD-Growth Arrest and DNA Damage
GAPPS	Gastric Adenocarcinoma and Proximal Polyposis of the Stomach
GATA1	Globin transcription factor 1
GC	Gastric Cancer
GCL	Glutamate-cysteine ligase
GGR	Globale Genome Repair
GOJ	Gastroesophagal junction cancer
GR	Glutathione Reductase
GS	Genome Stable tumors
GSH	Glutathione Enzyme
GSK3B	Glycogen Synthase Kinase 3B
GST	Glutathione-S-Transferase
GW182	Glycine-tryptophan repeat-containing protein of 182 kDa
H	Histone
H2S	Hydrogen sulphide

HAT	Histone AcetylTransferase
HDAC	Histone DeAcetylase
HDACi	Histone DeAcetylase inhibitor
HDGC	Hereditary Diffuse Gastric Cancer
HDM	Histone DeMethylase
HER2	Erb-B2 receptor tyrosine kinase 2
HIF1a	Hypoxia Inducible Factor 1 alpha
HMG	High Mobility Group
HMGA2	High mobility group AT-hook protein 2
HMGB	High Mobility Group Box
HMT	Histone MethylTransferase
HOXA5	Homeobox A5
HP1	Heterochromatin Protein 1
HPV-E6	Human PapillomaVirus-E6
HSP90	Heat Shock Protein 90
hTERT	Human Telomerase Reverse Transcriptase
IARC	International Agency for Research on Cancer
IGC	Intestinal Gastric Cancer
IL8/2/6/1	Interleukine
IM	Intestinal Metaplasia
IRE1a	Inositol-Requiring Protein 1a
ISR	Integrated Stress Response
ISWI	Imitation SWI of drosophila melanogaster
ITCH	Itchy E3 ubiquitin protein ligase
JAK2	Janus Kinase 2
JNK	c-Jun N-terminal Kinases
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LMP2A	Latent Membrane Protein 2A
MAP4	Microtubule-Associated Protein 4
MAPK	Mitogen-Activated Protein Kinases
MBP	Methyl-CpG Binding Protein
MDB	Methyl-CpG-Binding Domain
MDM2	Mouse Double Minute 2 homolog
MeCP2	Methyl-CpG Binding Protein 2
MEF	Mouse Embryonic Fibroblast

MEF2	Myocyte Enhancer Factor 2
miRNA	micro-RNA
MLH1/2	MutL Homolog
MMP	Metalloproteinase
MMR	Mismatch Repair
MPT	Mitochondrial Permeability Transition
mRNA	Messenger RNA
MSH2/6	human MutS Homolog 2
MSI	Microsatellite unstable tumors
mSin3A	Paired amphipathic helix protein
mTOR	mammalian Target of Rapamycin
MYCN	Neuroblastoma MYC Oncogene
NaB	Sodium Butyrate
NAD	Nicotinamide adenine dinucleotide
NCR	Nuclear receptor Co-Repressor
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NER	Nucleotide Excision Repair
NES	Nuclear Export Sequence
NFR	Nucleosome Free Region
Nk-kB	Nuclear Factor Kappa B Subunit 1
NLS	Nuclear Localization Sequence
NQO1	NAD(P)H Dehydrogenase Quinone 1
NSCLC	Non-Small Cell Lung Cancer
NuRD	Nucleosome Remodeling Deacetylase
OS	Overall Survival
PACT	Protein ACTivator of the interferon-induced protein kinase
PCAF	P300/CBP-Associated Factor
PCNA	Proliferating Cell Nuclear Antigen
PD-L1/2	Programmed Death-Ligand
PDI	Protein Disulfide Isomerase
PERK	Protein Kinase RNA-like ER Kinase
PFS	Progression Free Survival
PI3K/Akt	PhosphatidylInositol-3-Kinase/Akt
PIK3CA	PhosphatidylInositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
Pin1	Prolyl Isomerase

Piwil2	Piwi-like protein 2
PKC	Protein Kinase C
PKR	Interferon induced ds-RNA activated protein kinase receptor
PLZ	PML-Zinc finger
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced protein 1
PML	Promyelotic Leukemia
PMS2	Post-Meiotic Segregation 2
Pol II/III	RNA Polymerase
PPAR	Peroxisome proliferator-activated receptor
PPI	Proton pump inhibitors
PTA	1,3,5-Triaza-7-phosphaadamantane
PTEN	Phosphatase and TENsin homolog
R-SMAD	Receptor-SMAD
RA	Retinoic acid
Ran-GTP	Ras-Related Nuclear Protein-Guanosine-5'-triphosphate
RAR	Retinoic Acid Receptor
Rb	Retinoblastoma
RbBP	Retinoblastoma Binding Protein
RDC	Ruthenium Derived Compounds
RE	Response Element
RHOA	Ras homolog gene family, member A
RISC	RNA induced silencing complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RPB3	RNA Polymerase B
SabA	Sialic acid–binding adhesin
SAC	Spindle Assembly Checkpoint
SAHA	Suberoylanilide hydroxamic acid
SAM	S-Adenosyl-L-methionine
SHP2	Tyrosine-protein phosphatase non-receptor type 11
SIRT1	Sirtuin 1
SMAD	Mothers Against DPP Homolog 1
SOD	Superoxide Dismutase
SP1	Specificity Protein 1

SRY	Sex-determining Region of Y chromosome
STAT1/3	Signal Transducer and Activator of Transcription 3
SUMO1	Small Ubiquitin-Like Modifier 1
SWI/SNF	SWItch/Sucrose Non-Fermentable
SWR1	SWi2/snf2-Related
T4SS	Type IV Secretion System
TCGA	The Cancer Genome Atlas
TCR	Transcription-Doubled Repair
TFIIH	Transcription Factor II H
TGFB1	Transforming Growth Factor Beta 1
TLR	Toll-Like Receptor
TMEM205	Transmembrane protein 205
TNFa	Tumor Necrosis Factor a
TRB3	Tribbles Pseudokinase 3
TRBP	TAR RNA-Binding protein
TRIM32	Tripartite motif-containing protein 32.
TSA	Trichostation A
TSS	Transcription Start Site
UBF	Upstream Binding Factor
UFD2a	Ubiquitination Factor E4B
UPR	Unfold Protein Response
UTR	Untranslated Region
VacA	Vacuolating cytotoxin A
VDAC	Voltage Dependent Anion Chanel
VEGF	Vascular endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	Von Hippel–Lindau
VPA	Valproic Acid
WHO	World Health Organisation
XBP1	X-box-Binding Protein 1
XPA	Xeroderma Pigmentosum complementation group A
YAP1	Yes Associated Protein
YY1	Yin Yang 1
ZEB1	Zinc Finger E-Box Binding Homeobox 1
Zn	Zinc

INTRODUCTION

I. GASTRIC CANCER

1. Epidemiology

Almost one million new cases of gastric cancer (GC) were diagnosed in 2012 (952,000 cases, 6.8% of the total), making it the fifth most common malignancy in the world, after lung, breast, colorectal and prostate cancers according to the World health organisation (WHO) database (*Mihmanli and al., 2016*). This represents a decrease since the very first estimates in 1975 when GC was the most common cancer, but survival rates remains low. There is a disparity in the geographical distribution and socio-economic groups of GC with the highest incidence in Eastern Asia (Korea, Mongolia, Japan, China), Central and Eastern Europe and South America (**Figure 1;** *Crew and al., 2006*). Indeed, more than 70% of cases occur in developing countries, and half the world total occurs in Eastern Asia (mainly in China). GC is two times more common in men than in women and it represents the third leading cause of cancer death in both sexes worldwide (723,000 deaths, 8.8% of the total), due to a late diagnosis and the limited range of treatment options *(Torre and al., 2015)*.
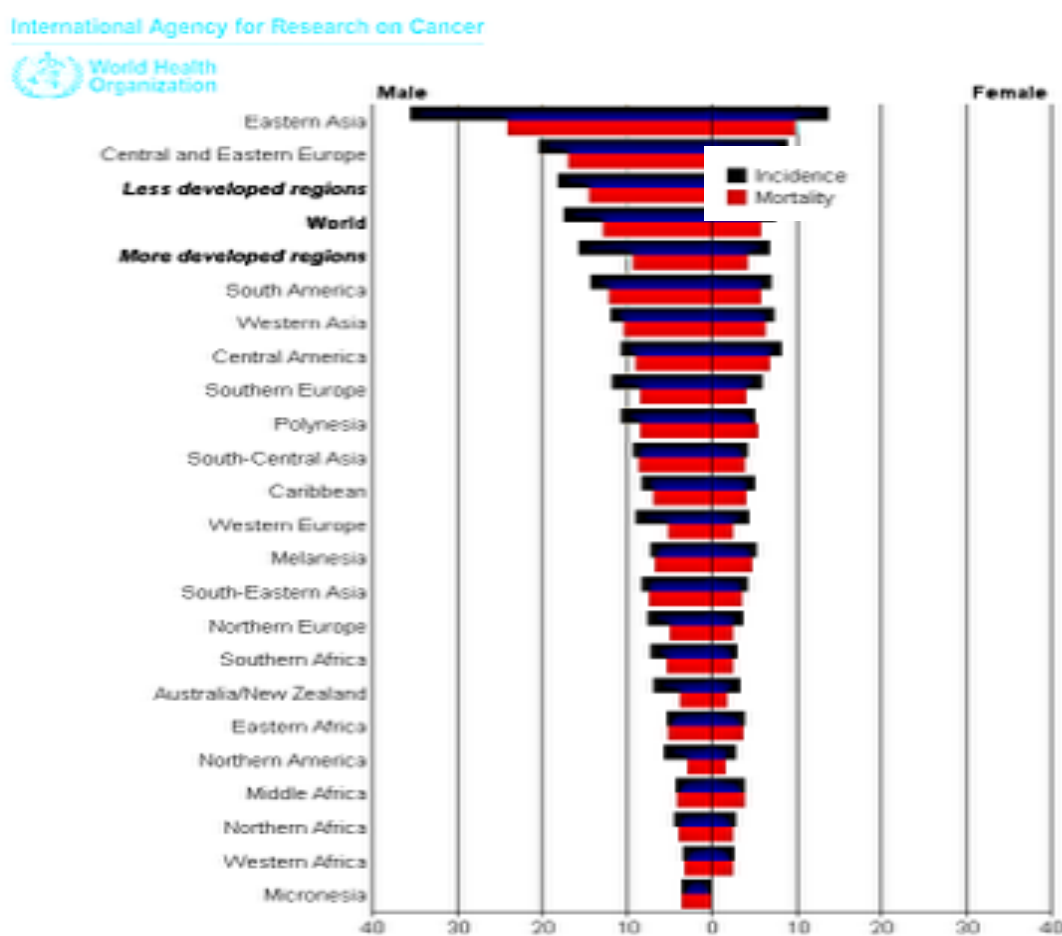
Figure 1: GLOBOCAN, Estimated Incidence, Mortality and Prevalence Worldwide in 2012

In GC, two different topographies can be distinguished: the distal cancer reaching the pyloric region, the antrum, body, or fundus, and the proximal cancer reaching the cardia region *(Karimi and al., 2014)*. Incidence rates and risk factors showed differences according to tumor types, with upward trends in cardia GC type incidence *(Alberts and al., 2003)*. In fact, in developing countries, GC is mostly related to the infection by *Helicobacter pylori* with intestinal histology type. Conversely, in Eastern Europe and USA, there are more diffuse GC from cardia region that are more aggressive and difficult to treat *(Pacelli and al., 2001)*. It is precisely the latter type of GC which is increasing in developed countries. For example, in France it represents 4500 deaths per year due in part to lack of efficacy of current treatments (**Figure 2**).
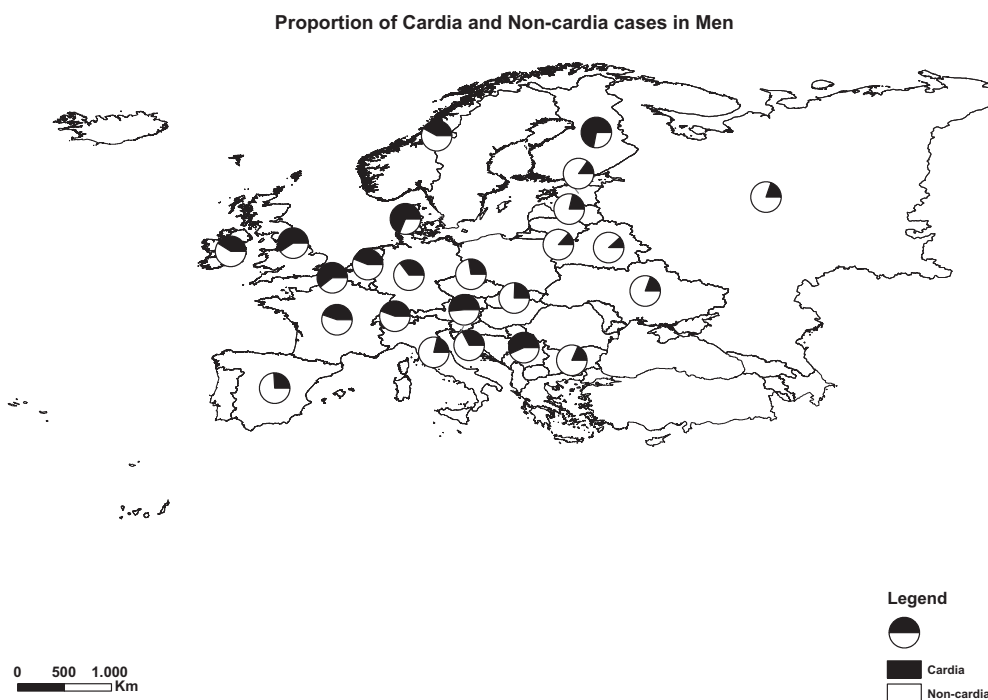
Figure 2: Proportion of cardia and non-cardia gastric cancers in men in selected European countries. *Ferrero and al., 2014*

Decrease of distal GC during the last decade can be explained by a better management of the infection of *Helicobacter pylori*, a better diet (fresh fruits and vegetables), a decrease in tobacco and salt consumption. Conversely, increase in proximal GC type is due to a rise in obesity, acidic reflux and a better classification of the cancer (*Terry and al., 2002*; ***see risk factors section***).

2. Classifications of gastric carcinoma

Around 95% of GC are adenocarcinoma and the others are lymphoma (4%) and malignant gastrointestinal stromal tumors (GIST, 1%) (*Kumar and al., 2013*). Several classifications exists, GC can be clinically classified as a true GC in an early and advanced stage or a grastroesophagal junction adenocarcinoma to help determine appropriate intervention, and histologically into subtypes based on major morphologic component. In 50 years of systematic classification of the GC pathohistological characteristics, there is no sole classification system that is consistently used worldwide in diagnostics and research (*Berlth and al., 2014*).

2.1. Clinical classifications

The stomach is divided into several anatomic subsites, including the cardia (top inch of the stomach), fundus, body, so-called the proximal site and the pylorus, and the antrum, the distal site. These areas are distinguished by anatomic demarcations and histological differences (**Figure 3**; *American Cancer Society*). It is important to discriminate cancers arising from the cardia region including those from the gastroesophagal junction (GOJ) and those coming from the other parts of the stomach (non-cardia GC), because they have different epidemiologic patterns and causes (*Karimi et al, 2014; Forman and al., 2006*).

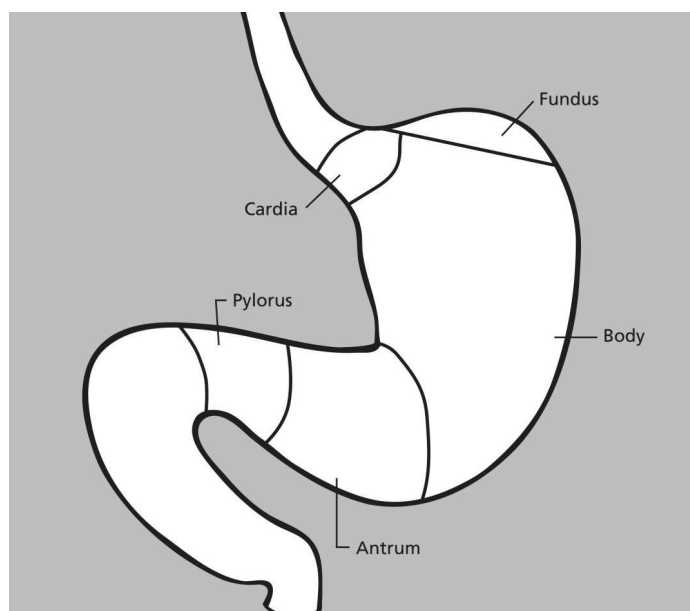**Figure 3: Anatomical disposition of stomach,** *American Cancer Society*

2.1.1. Cancers at gastric cardia region and gastroesophageal junction

It is noteworthy that, many studies are trying to define criteria to classified tumors arising from the cardia region of the stomach especially if they involve the GOJ and the distal oesophagus, into GC

or oesophagus cancer. As yet, there is no universal guideline regarding the anatomic definition of gastric cardia (*Hayakawa and al., 2016; Wittekind, 2015*). To improve this classification, the American Joint Committee in collaboration with the Japan Gastric Cancer Committee reviewed the TNM classification of GC. Tumor node metastasis (TNM) staging provides guidance for selecting the optimal treatment modalities. It also provides information on the prognosis for both clinicians and patients. Grossly, this classification is based on the location of the tumor epicenter (plus or minus 5cm) and the presence or absence of GOJ involvement (*Biondi and al., 2011*). Unfortunately, many groups have conducted retrospective studies, and shown that this simplified classification still required improvements. Indeed according to them, it would be easier to classify these tumors in the GC setting rather than in the oesophagus cancer (*Röcken and al., 2015*). To improve the classification and treatment, we need more knowledge about the cardia GC.

2.1.2. Early and advanced gastric carcinoma

Early gastric carcinoma (EGC)

As described by Hamilton et al., EGC is defined as invasive carcinoma confined to mucosa and/or submucosa, with or without lymph node metastasis, irrespective of the tumor size (*Hamilton and al., 2000*). It has been identified in Japan where there is active screening of patients at high-risk for GC. The prognosis of EGC is excellent with a 5 years survival around 90%. It is divided into four types according to the macroscopic growth patterns of the mucosal surface. Briefly, this classification described tumors with: (**figure 4;** *Carl-Mcgrath and al., 2007*)

- **Type I** - protruding growth

- **Type II** - superficial growth, which is further divided into three subtype as proposed by the Japanese Endoscopic Society : *Type IIa* - elevated; *Type IIb* - flat; *Type IIc* - depressed

- **Type III** - excavating growth

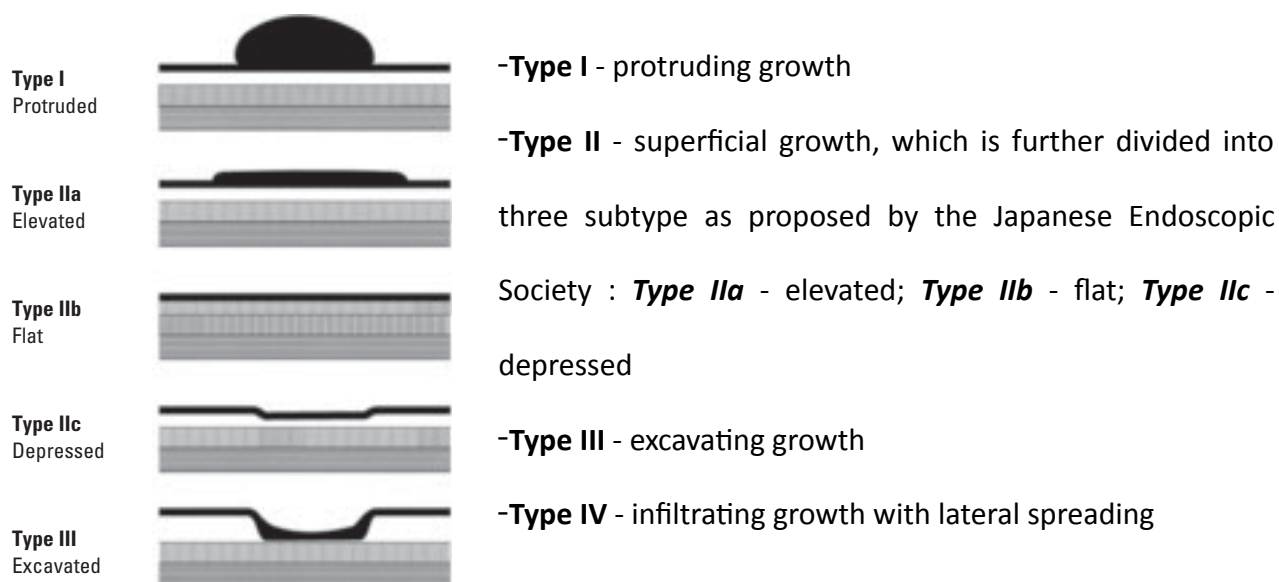- **Type IV** - infiltrating growth with lateral spreading

Figure 4: Growth features of Early gastric carcinoma, *Hamilton and al., 2000*.

Advanced gastric carcinoma (AGC)

In contrast, AGC invades into muscular propria or beyond and correlated with a worse prognosis with a 5 years survival less than 60%. Based on Borrmann's classification, it is divided into four subtypes, also according to the gross appearance of the mucosal surface (**Figure 5**; *Japanese Research Society for Gastric cancer, 1998*):
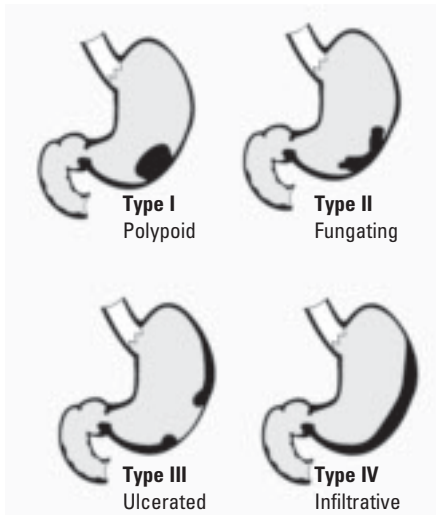
-**Type I** - polypoid growth

-**Type II** - fungating growth

-**Type III** - ulcerating growth

-**Type IV** - diffusely infiltrating growth which also refer to linitis plastica in signet ring cell carcinoma when most of gastric wall is involved by infiltrating tumor cells.

Figure 5: Bormann's classification of advanced gastric carcinoma, *Hamilton and al., 2000.*

The distinction between EGC and AGC before resection is clinically important because it helps decide the pre-operative therapy which has shown to improve disease free survival and overall survival.

2.2. Histological classifications

There are various systems applied to the histological classifications: the WHO, Ming, Laurén and Goseki classifications. Some proposed classifications are based on clinical and endoscopic appearances of the tumors, some on histopathology alone. The clinical significance of these classifications is limited, with only the Laurén and the WHO classifications referred by several national guidelines for GC treatments *(Berlth and al., 2014)*.

2.2.1. Lauren classification

However, the Lauren classification, described in 1965, has been the most successful system (*Lauren, 1965*). It defines two distinct histological types by microscopic morphology alone: the intestinal type, and the diffuse type, which appears clearly as dissimilar clinical and epidemiological entities. Later the indeterminate type was included to described non common

histology *(Ma and al., 2016; Chen and al., 2016a)*. The relative frequencies are approximately 54% for intestinal type, 32% of diffuse type and 15% of the indeterminate type *(Hu and al., 2012).*

- **Intestinal type (IGC) -** Tumor cells are well-differentiated, adherent, and exhibit tubular and glandular formations. IGC are always associated with intestinal metaplasia and exhibit lymphatic and vascular invasion, with scattered lesions. IGC are located into the antrum of the stomach and are more often observed in elderly men than in women and correlated with a better outcome for patients *(Roukos and al., 2002a)*.

- **Diffuse type (DGC)** - Tumor cells are undifferentiated, and exhibit stromal invasion as a mucocellular single cell or subgroup, the linitis plastica. They form a non cohesive population with scattered tumoral cells. Metastasis development appears faster than in the IGC, with peritoneal and lymphatic localisations. It affects the body region of the stomach. There is a prevalence for both men and women and it tends to occur in younger people than the IGC, with a worse prognosis for patients *(Vauhkonen and al., 2006)*.

- **Indeterminate type** - including mixed tumors with intestinal and diffuse characteristics.

2.2.2. WHO classification

The World Health Organization (WHO) classification appears to be the most detailed among all the pathohistological classification systems. It includes not only adenocarcinoma of the stomach but also all other types of gastric tumors of lower frequencies, equivalent to the indeterminate type in Laurén classification *(Fléjou, 2011)*. So the WHO classification is more used for the infrequent types of GC. It is based on the predominant histologic pattern of the carcinoma which often co-exists with less dominant elements of other histological patterns. The 2010 WHO classification divides gastric tumors in 5 subtypes *(Werner and al., 2001; Chen and al., 1994; Qui and al., 2013).*

- **Tubular adenocarcinoma** - Irregular-shaped and fused neoplastic glands with intraluminal mucus and debris. It is the most common histologic type of EGC. A poorly differentiated variant is sometimes called solid carcinoma.

- **Papillary adenocarcinoma** - Epithelial projections scaffolded by central fibrovascular core. It occurs in the proximal region of the stomach and tends to affect elderly patients.

- **Mucinous adenocarcinoma** - Clusters and scattered tumor cells floating in the abundant extracellular mucin. By definition, more than 50% of extracellular mucin is retained within the tumor, and called the mucinous pools.

- **Signet-ring cell carcinoma (including poorly cohesive carcinoma)** - More than 50% of the tumor consists of isolated or small groups of malignant cells containing intracytoplasmic mucin.

- **Mixed carcinoma** - It consists of a mixture of different types of gastric adenocarcinoma.

The well-differentiated tubular and poorly differentiated signet-ring cell carcinoma make up the majority of tumors and they can be linked with the IGC and DGC respectively described in the Laurèn classification.

3.Pathogenesis

3.1. Precancerous cascade

Gastric carcinogenesis is described as a multistep and multifactorial process by Correa *(Correa and al., 1975)*. GC is define to be the end-result of an inflammatory process explained by environmental factor, food intake or bacterial / virus infection. Its progression leads a sequential chain of events, the precancerous cascade or « Correa sequence » *(Correa and al., 2007; Siurala and al., 1966; Rugge and al., 2000)* characterized as:

Chronic Nonatrophic Gastritis

Mucosal infiltration by white blood cells (lymphocytes, plasma cells, macrophages) with a prevalence in the antrum part of the stomach. This step can also be found in the case of a duodenal ulcer, which show no progression into GC in most of case.

Multifocal Atrophic Gastritis

Loss of stomach glands, like the oxyntic glands composed of the parietal cells responsible to the production of hydrochloride acid. So the stomach lacks its capacity to produce gastric acid, leading to a higher bacterial colonization. It represents the critical step into the progression of gastric cancer.

Intestinal metaplasia (IM) complete and incomplete

In younger patients the complete type of IM predominates, defines by the replacement of the normal lining of the stomach by cells usually found in the small intestine (absorptive enterocytes, brush border, microvilli). Older patients tends to have incomplete IM, which metaplastic cells lose their small intestine phenotypes and acquire morphologic features of the large intestine and are lined only by goblets cells expressing gastric and intestinal mucin.

Dysplasia

Atypical changes in nuclear morphology and tissue architecture, which is irregular, frequently forming packed tubular structures (adenomas) with irregular lumens. The cells are confined within the tubular structures. As the invasive parts are often missed by biopsy, they are classified into low and high grades depending on the degree of atypical changes.

Invasive carcinoma

Dysplasic cells go through the basal membrane to become invasive carcinoma.

The "Correa sequence" is mostly related to the IGC type, combined with the *H.pylori* infection and others environmental factors. In the case of the DGC type, it is more complicated, as it originates from gastric mucosa and it is associated with gastritis. Unfortunately, because of a delayed in the diagnosis resulting in an advanced stage of the disease, there is a lack in well-recognized precursor changes leading to its progression *(Correa and al., 1994)*. Moreover, DGC seems to be less affected by the environmental factors, even if the *H.pylori* infection can be involved.

3.2. Risk factors

Gastric cancer is a multifactorial disease composed of both environmental and genetic factors. The marked geographic variations, time trends and the migratory effect on GC incidence suggest that environmental and lifestyle factors are major contributes to the etiology of the disease *(Ushijima and al.,2004)*.

3.2.1. *Helicobacter pylori* infection

Helicobacter pylori (H.pylori) is associated with a six fold increased risk of GC, leading to its definition as a human class I carcinogen by the International Agency for Research on Cancer (IARC) in 1994 *(Crew and al., 2006)*. *H. pylori* represents the most common chronic bacterial infection worldwide, infecting more than 50% of the world population *(Fox and al., 2007)*. It is a Gram negative bacillus which is known to colonize the gastrointestinal system, specially the stomach mucosa *(Marshall and al., 1984)*. *H. pylori* infection prevalence increases with age and is found to be higher in the developing countries with a lower socioeconomic status, and so linked to the strongest incidence of the non cardia GC. Conversely, in the developed countries there is a decrease in the infection by *H. pylori* and the non cardia GC incidence, which can be explained by a better sanitation and an increase in antibiotics intake. The majority of people are infected during childhood and are asymptomatic, the infection persists throughout life *(Feldman and al., 2001)*.

The infection by *H. pylori* is not sufficient to induce GC alone. Indeed, only 10 to 15% of the infected population develops a peptic ulcer and only 1 to 3% of patients develop a GC *(Taylor and al., 1995)*.

In the "Correa sequence", *H. pylori* is known to initiate the chronic gastritis and to be associated with the development of the atrophy and the intestinal metaplasia. There are different pathways that can explain its effect on gastric mucosa *(Correa and al., 2007)*:

Acidic resistance of *H. pylori*

The pH of the gastric mucosa is between 4 to 6,5. *H.pylori* is not an acidophilic bacterium, so to survive and colonize the stomach it secrets some proteolytic enzymes as the urease, which hydrolyzes urea in ammonium, bicarbonate and carbone dioxide able to decrease gastric acid pH. Moreover, ammonium is known to have a cytotoxic effect on epithelial cells and the bicarbonate can suppress the bactericide effect of some nitric oxide metabolite present in the stomach *(Oh and al., 2005; Wilkinson and al., 1998)*.

***H.pylori* virulence factors : CagA and VacA.**

The ***CagA factor*** is expressed in 50-80% of GC. It is mostly found in patients with a IGC type. The genomic regions, *cag* pathogenicity island (*cag* PAI), encoded for CagA and a type IV secretion system (T4SS). T4SS is required for the translocation of CagA into the host cells. Then, it can be phosphorylated by host kinase (Src, Abl), a crucial step leading to its interaction with the tyrosine phosphatase SHP-2 to act on the morphological rearrangement of the epithelial cells and cell proliferation. Moreover, CagA can suppress the expression of p53 in a p14ARF-dependent manner *(Wei and al., 2015)*. p14ARF is a tumor suppressor upstream of p53, allowing its accumulation under stress conditions. Through direct interaction with MDM2 and ARF-BP1, two ubiquitin ligases responsible of p53 proteasomal degradation, it allows their sequestration in the nucleus and inhibition of their E3 ligase activity *(Chen and al., 2006).* p14ARF, as p53, is frequently inactivated during the progression of the GC by promoter hypermethylation or deletion *(Sato and al., 2006).* CagA could also have an effect on the induction of some pro-inflammatory modulators like the interleukin 8 (IL8). The non-phosphorylated CagA can elicit disruption of tight and adherent junctions, loss of cell polarity, pro-inflammatory and mitogenic response *(Higashi and al., 2002; Tsutsumi and al., 2003)*.

The ***VacA factor (vacuolating cytotoxin)*** is secreted by approximately 50% of *H.pylori* strains and has a vacuolating effect on gastric cell lines in vitro. It can form pores in epithelial cell membranes inducing the release of urea and nutrients *(Leunk and al., 1988)*. Moreover, this factor has a pro-apoptotic cell-dependent effect mostly in parietal cells, leading to a decrease in acid secretion. It

can block the proliferation of the immune T-cell by a cell cycle arrest in the G1-S phase leading to a decrease in the downstream immune signalisation of TLR and IL2 pathway, important for pathogen recognition. This mechanism can explain the persistence of the infection *(Gebert and al., 2003)*.

Adhesin and outer membrane proteins

Only 10% of *H. pylori* can bind directly to the epithelial cells, which implicates numerous bacterial cell-surface receptors *(Semino-Mora and al., 2003)*. Among them, there is the Lewis-b binding adhesin (BabA), an outer membrane protein, which can interact with the Lewis B histo-blood group on the host cells. The same mechanism implicates the receptor SabA and the host protein Lewis X, a glycophospholipid, which is a known tumoral antigen used as a biomarkers for GC dysplasia. *H. pylori* strain positive for BabA, CagA and VacA present a higher risk to develop GC for patients *(Backstrom and al., 2004; Gerhard and al., 1999).*

Contribution of host genetics

The risk to develop a GC in presence of *H.pylori* infection can be higher when there is an increase in the level of pro-inflammatory host factors, for example in the case of genetic predisposition for sporadic DGC, like the polymorphism of pro-inflammatory IL1B gene, a known inhibitor of acid secretion. This leads to its high-level expression and so a reduced acid out-put associated with an increase in *H.pylori* colonization *(El-Omar and al., 2000)*. Similar effects have been observed with the pro-inflammatory cytokine TNFα and IL1 influencing gastrin production by the parietal cells. Moreover, polymorphism in the IL10 gene leads to a strongest immune response and so an increase in the development of the atrophic gastritis *(El-Omar and al., 2003)*.

3.2.2. Others risk factors

Epstein-Barr virus (EBV)

EBV, also known as human herpes virus type 4, is a gammaherpesvirus and thus has a double stranded DNA genome. It is found in malignant cells but not in the normal epithelial cells. The infection does not present symptoms in 90% of cases and is associated with only 2% of all gastric cancer tumors. So as *H. pylori*, it was defined as a type I carcinogen by the IARC. Originally, EBV was described particularly for its association in the initiation and progression of lymphomas and nasopharyngeal carcinomas (mainly in China population; *IARC, 1997*).

EBV-associated GC has been described in the 90s, affecting 9% of tumors. It is a monoclonal infection, indicating that the tumor arises from a single infected cell. It has so far no histological characteristics *(Oh and al., 2004)*. However, EBV-positive tumors seem to have a strong lymphatic

infiltration *(Kelley and al., 2003)*. A higher prevalence in men is observed compared to women, with an equivalent achievement in IGC and DGC. EBV-associated GC is mostly found in the fundus and corpus of the stomach. The mechanisms involved in the initiation and progression of CG are currently unclear, however, it has mostly been described for its molecular characteristics. Indeed, EBV positive tumors present a higher hypermethylated status compare to the EBV negative tumors (*Network TGCA research, 2014;* see **Molecular Characterization of GC**)

Dietary

Diet-related risk factors are mostly found in non-cardia IGC type. The consumption of fresh vegetables and fruits is inversely correlated with the risk of developing GC. Indeed, the reduction in the incidence of non-cardia cancer is partly explained by the advent of the refrigerator and a better diet in developed countries. In addition, these foods are known to contain carotenoids, vitamin C or selenium, compounds known for their anti-oxidant property favorable to the risk reduction of developing cancer of the digestive system *(Bjelakovic and al., 2004)*.

Moreover, excessive consumption of salt and salty food is shown to be strongly associated with the development of GC, especially during *H. pylori* infection. Indeed salty foods constitute a significant source of nitrites and nitrosamines. Nitrates will be derived into N-nitroso compounds, known to be carcinogenic, by nitrosating bacteria via bacterial nitrosation. The development of these bacteria is promoted in the presence of *H. pylori (Liu and al., 2008)*.

Others risk factors

Tobacco consumption is strongly associated with the development of GC *(Gonzalez and al., 2003)*. Indeed, it was shown in a ferret animal model to be linked to a decrease in the p53 phosphorylation, a well known tumor suppressor particularly described to be inactivated in the GC. Thus, it leads to a reduction of some of its target genes such as *BAX*, *p21*, and so an impact on cell proliferation and apoptosis *(el-Deiry and al., 1998)*. Some studies have shown a synergy between the consumption of tobacco and alcohol even if the results remain controversial until now *(Sjodahl and al., 2007)*.

Patients overweight and obesity present a risk factor for the development of GC, especially it seems to play an important role for the cardia diffuse type GC. This factor is often associated with the presence of the gastroesophagal reflux in patients *(Hoyo and al., 2012)*.

Finally, there is some genetic predisposition for the development of GC. I have already mentioned the polymorphism of the IL1B associated with *H.pylori* infection. In addition, people with a type A blood rhesus, a pernicious anemia, a family history, a hereditary nonpolyposis colon cancer, a Li-

Fraumeni (inherited p53 mutation) or Lynch syndrome also present a higher risk of developing GC *(Roder and al., 2002; Nomura and al., 1996)*.

In conclusion, most of the described risk factors are associated with the non-cardia IGC type. For the cardia DGC type, *H.pylori* and overweight or obesity are commonly found.

3.3. Hereditary syndromes

Only 10% of GC are related to GC familial cluster, and 1-3% of this cases have specific genetic patterns *(Oliveira and al., 2009)*. There are three types of familial hereditary GC:

Hereditary DGC (HDGC)

This is the only case of familial GC with a determined genetic cause. The penetrance of HDGC is more than 80% into a family. Indeed, for 40% of patients there is a germline heterozygous mutation inherited in the gene *CDH1* (E-cadherin). This mutation leads to a deleterious conformational change of the protein that causes its first hit inactivation *(Corso and al., 2012)*. Somatic molecular events can also lead to a second hit inactivation of the second allele, mainly due to the promoter hypermethylation *(Ushijima and al., 2004)*. In 60% of cases, we do not observe gene mutation but germline phenotype of monoallelic *CDH1* downregulation *(Pinheiro and al., 2010)*. Inactivation or decreased expression of E-cadherin, an important surface protein in the maintenance of intercellular junctions, will allow an increase in the migration and cell invasion. Furthermore, studies have recently shown the involvement of the truncated mutation in *CTNNA1* gene (alpha-E-catenin), in combination or not with *CDH1* mutation *(Majewski and al., 2013)*.

Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS)

GAPPS have been described for the first time in 2012. It is an autosomal dominant transmission of fundic gland polyposis at the proximal site without evidence of colorectal or duodenal polyps, or other hereditary gastrointestinal cancer syndrome. The penetrance of GAPPS is incomplete and the genetic causes are currently not known *(Oliveira and al., 2015; Worthley and al., 2012)*.

Familial IGC (FIGC)

FIGC is an autosomal dominant inheritance with an IGC type. As for the GAPPS, no information is available for the genetic causes *(Oliveira and al., 2015; Caldas and al., 1999)*.

4. Molecular characterizations of GC

Everyone agrees on the fact that GC is a heterogeneous cancer. It does not only present inter-patient heterogeneity but also and especially intra-tumoral heterogeneity. Through the various existing clinical and histological classifications for this cancer, we can see a real complexity in terms of definition of the various existing subtypes. Moreover, this complexity results in a lack of information about patient survival and the choice of the most relevant treatment *(Lin and al., 2015; Chen and al., 2016b)*.

These last years, with the emergence of high-throughput experiments, several teams tried to stratify the GC based on their molecular characteristics. Indeed studies have been conducted to define the molecular signature of the GC to improve its diagnosis, to define better subtypes, and to predict the TNM status, the response to chemotherapy or the prognosis *(Cristescu and al., 2015)*. Unfortunately the recurring problems of all these studies are the insufficient number of samples analyzed and the variation of the treatments used. I am therefore interested in two studies conducted by the Asian Cancer Research Group and the Cancer Genome Atlas, which reflect the geographic disparity of the incidence rate of GC, allowing a more accurate analysis *(Lin and al., 2015a; Cancer Genome Atlas Research Network., 2014)*.

4.1. The Cancer Genome Atlas (TCGA) molecular classification

In 2014, TCGA defines a new molecular characterization, integrating the genetic alteration with fresh biopsy from 295 primary gastric adenocarcinomas, many from the United states and Western Europe. The patients were not treated with chemotherapy or radiotherapy. The project was based on the molecular characterization of GC by using six different platforms and related the results with the common histological classification. They define four different subtypes for GC, the EBV positive tumors (EBV-GC), the microsatellite unstable tumors (MSI), the genome stable tumors (GS) and the chromosomal instability tumors (CIN) (**figure 6**). The IGC type was found across all subtypes, conversely the DGC type was mostly linked with the GS type *(Bass and al., 2015; Zhang and al., 2014a; Network TCGA research, 2014)*.

The Epstein-Barr virus-associated tumors (EBV-GC)

As mentioned earlier, the EBV positive tumors present an extreme CpG island methylator phenotype *(Kaneda and al., 2012)*. Indeed the EBV viral protein LMP2A will allow the phosphorylation of STAT3 thus leading to the transcription of the DNA methyltransferase 1 (DNMT1) and the loss of certain gene expression such as *CDKN2A* (p16) and *P73*. In addition, it is

also found that 50% of tumors harboring mutations such as PIK3CA (subunit of PI3K/Akt pathway), ARID1A, BCL6 and rarely p53 mutation. Furthermore, there is also gene amplifications such as JAK2, which plays an important role in the signaling of some cytokines such as prolactin, or amplification of PD-L1 and PD-L2 important in the control of the immune system via T-cell inhibition.

The microsatellite unstable tumors (MSI)

The MSI occur at an early stage in GC progression and were found in 21% of cases. These tumors are characterized by the loss of expression of MLH1 by the promoter hypermethylation, an important enzyme in the mismatch repair in response to DNA damage. There is a hypermutation state of those tumors with mutation of some genes like HER2, PIK3CA or EGFR but no gene amplification is found *(Oda and al., 2005)*.

The genomically stable tumors (GS)

The GS are found in 19% of cases especially in the DGC type. They are characterized by the mutation of *RHOA* gene playing a role in cell motility and altering cell adhesion. *CDH1* gene mutation is also present in these tumors, which is consistent with previous studies linking its inactivation with DGC type. Interchromosomal translocations are observed leading to the fusion of the claudin 18 gene and the Rho-GTPase activating protein 6 gene. This fusion protein will have a pro-oncogenic role in the growth and tumor prognosis.

The chromosomal instability tumors (CIN)

The CIN are the result of an unequal distribution of DNA in the daughter cell during mitosis, resulting in chromosomal gain or loss, such as the chromosome 8q or 3p respectively *(Tsukamoto and al., 2008)*. In addition, CIN contribute to the amplification of some gene like the receptor tyrosine kinases (EGFR), or some modulators of the cell cycle (cyclin E, D1, CDK6) *(Weiss and al., 2004)*.
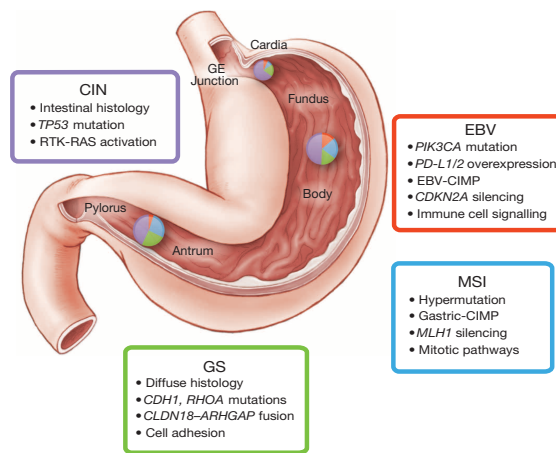
Figure 6: Key features of gastric cancer subtypes. This schematic lists some of the salient features associated with each of the four molecular subtypes of GC. Distribution of molecular subtypes in tumours obtained from distinct regions of the stomach is represented by inset charts, *Network TGCA research, 2014.*

4.2. Asian Cancer Research Group (ACRG) molecular classification

Another molecular classification was done by the ACRG in 2015 with 300 Korean GC tumors using the same approach. Most of the tumors are DGC type. They also find a MSI subtype, but not a distinct EBV subtype as described before by the TCGA group. In their study, the subtype mostly linked to DGC type was characterized by a gene signature of the epithelial-mesenchymal transition. Moreover to discriminate the other tumors they chose the gene *TP53*, known to be inactivated in 60% of GC (**figure 7**). Briefly the 4 molecular subtypes for GC defined by the ACRG are defined by (*Lin and al., 2015a; Lin and al., 2015b; Critescu and al., 2015)*:

The microsatellite stable (MSS) / Epithelial-Mesenchymal Transition (EMT) tumors
The MSS / EMT tumors occurs at an elderly age and are correlated with a poor prognosis. They are mostly of diffuse type with a high frequency of recurrence (63%). They have as a major characteristic, the loss of *CDH1* gene expression, which can be observed in the GS tumor of TCGA classification.

The microsatellite instability tumors (MSI)
The characteristics of MSI are equivalent with those of TCGA classification, i.e. hypermutation and the loss of MLH1 expression *(Mori and al., 2003)*. They are mainly located at the antrum site and correlated with a better prognosis and a lower recurrence frequency (22%).

The MSS / TP53 positive (p53+) tumors

Most of the p53+ tumors are positive for EBV and so correlated with a cytokine gene expression signature. Moreover, an enrichment of PIK3CA and ARID1A mutations is observed. These tumors show an intermediate survival and recurrence in comparison with the EMT and MSI tumors with a better prognosis for patients compared to those with p53- tumors.

The MSS / TP53 negative (p53-) tumors

The p53- tumors display a high genetic instability with a strong focal gene amplification (EGFR, ERBB2, MYC) and p53 mutations in 60% of cases. Like for p53 + tumors they show an intermediate survival and recurrence.

In a more general way, the interest of the molecular characterization of GC has opened the search for new perspectives in the treatment field. Indeed, these patients will benefit of targeted therapy in function of the gene involved.

Lauren	GC			
	Diffuse type	**Intestinal type**		
TCGA	**GS**	**EBV**	**MSI**	**CIN**
	• *CDH1* and *RHOA* mutations • *CLDN18–ARHGAP26* fusion • Cell adhesion pathways • Younger patients	• DNA hypermethylation • *PIK3CA* mutation • *PDL1* and *PDL2* overexpression • Recurrent *JAK2* and *ERBB2* amplification • *CDKN2A* silencing • Immune cell signalling • Common in the corpus • Frequent *ARID1A* and *BCOR* mutation • Rare *TP53* mutation	• Hypermutation • *MLH1* silencing • KRAS or NRAS activation • *RASA1* and *PTEN* inactivation • Mitotic pathways • Older patients • Less A->C transversion	• RTK–RAS activation (*ERBB2*, *EGFR*, *MET*, *VEGFA* and *KRAS* or *NRAS*) • *TP53* mutation • Amplifications of cell cycle mediators (*CCNE1*, *CCND1* and *CDK6*), *GATA4* and *GATA6* • Common in GOJ and cardia cancer
ACRG	**MSS/EMT**	**MSS/*TP53*+**	**MSI**	**MSS/*TP53*−**
	• *CDH1* silencing • Younger patients • Worst prognosis	• Intact *TP53* • *MDM2* amplification • EBV infection • Enrichment with *PIK3CA* or *ARID1A* mutation and cytokine signature in EBV+ tumour	• Common in the antrum • Best prognosis • Hypermutation • *MLH1* silencing • Frequent mutations in *KRAS*, *MTOR*, *PTEN*, *PI3KCA*, *ASL* and *ARID1A*	• *TP53* mutation • Genomic instability • Recurrent amplification (*ERBB2*, *EGFR*, *GATA6*, *MYC*, *CCNE1* and *CCND1*) **Similar g**

Figure 7: Molecular subtypes of gastric cancer. Gastric cancer (GC) has four molecular subtypes characterized by specific genomic alterations: microsatellite instability (MSI), genome-stable (GS), Epstein–Barr virus (EBV) and chromosome instability (CIN) based on The Cancer Genome Atlas (TCGA) classification; and MSI, microsatellite stable (MSS)/epithelial–mesenchymal transition (EMT), MSS/TP53+ and MSS/TP53- based on the Asian Cancer Research Group (ACRG) classification. Diffuse-type GC is predominantly included in the GS or MSS/EMT subtype. It is likely

that MSI-type tumors represent classical intestinal-type GC in the distal stomach. CIN-type GCs are commonly found in the cardia. *Hayakawa and al., 2016*

5. Treatment

The diagnosis of patients is done by endoscopy and biopsy. Biopsies will enable clinical, histological and molecular classifications of the tumor, allowing the adaptation of the treatment *(Kumar and al., 2013)*. Unfortunately in Western countries, the diagnosis is delayed, because of an asymptomatic disease in the early stages, leading to a more advanced stage of GC. Moreover, unlike the Asian countries especially in Japan, we do not benefit from large screening program. So, the survival of patients with advanced GC and metastasis development is less than 20% in contrast to the 90% in EGC and AGC without metastasis development observed in Japan *(Alberts and al., 2003)*.

5.1. Curative treatment

Total or partial gastrectomy represents the cornerstone in the curative treatment for EGC and AGC without metastasis development. It is combined with chemotherapy or radio-chemotherapy *(Coccolini and al., 2016)*.

Surgery

The total or partial gastrectomy is associated with an adequate extend lymphadenectomy (at least 16 lymphatic nodes). Actually, there is a controversy about the extent of the lymph nodes resection. Indeed Japan surgeons favor an extended lymphadenectomy (D2) against a limited (D1) in European countries and in the USA. Despite a significant benefit in patient survival and relapse, a significant morbidity is observed in Western countries following the surgery explained by a lack of experience in the realization of D2 surgery for the Western countries *(Roukos and al., 2002b)*. The surgery is now commonly combined with chemotherapy or chemo-radiotherapy with a survival of 8-12 months compare to 3-4 months observed without chemotherapy *(Glimelius and al., 1997)*.

Chemotherapy and chemo-radiotherapy

Different protocols can be found in the treatment of GC. In fact either chemotherapy or chemo-radiotherapy is prescribed upstream (neoadjuvant) / downstream (adjuvant) the surgery or in a perioperative way. All these strategies have pros and cons. Indeed, neoadjuvant therapy shows a beneficial effect in reducing the tumor progression and possibly make surgery easier, but it

imposes a delay before the surgery that can affect the patient's survival and especially recurrence *(Schuhmacher and al., 2010)*. Adjuvant therapy also shows benefits for the overall survival (OS) and the progression free survival (PFS), first there is no delay for surgery and it can kill cancer cells too small to be detected during surgery *(Paoletti and al., 2010)*. Finally, perioperative therapy is frequently given in European countries in view of its profit on the OS, PFS and recurrence of GC *(Mirza and al., 2013)*.

According to the American cancer society guidelines, a number of anticancer drugs can be used to treat GC, for exemple 5-FU (fluorouracil), platinum derivatives (carboplatin, cisplatin, oxaliplatin) and Taxol derivatives (docetaxel, pacltaxel). These drugs may be used alone or in combination as:

- ECF (epirubicin, cisplatin, and 5-FU), which may be given before and after surgery
- Docetaxel or paclitaxel plus either 5-FU or capecitabine, combined with radiation as treatment before surgery
- Cisplatin plus either 5-FU or capecitabine, combined with radiation as treatment before surgery
- Paclitaxel and carboplatin, combined with radiation as treatment before surgery.

Targeted therapy

The new molecular classifications (TCGA and ACRG) allow putting into perspective the improvement of treatments with the use of targeted therapy. In fact, the molecular subtypes of GC have specific gene signatures, such as the *HER2 (Kurokawa and al., 2015)* and *VEGFR* gene amplification found for CIN or p53- GC types. Current treatments can target these amplifications with the use of humanized antibodies, Trastuzumab (*HER2*) and Beacizumab (*VEGFR*). Moreover targeted therapies are mainly used in combination of existing chemotherapies, and so they can reduce the toxicity of these treatments and improve patient survival. These last years numerous clinical trials have been introduced to determine the benefit of these targeted therapies in terms of PFS and OS in combination of current chemotherapies *(Bang and al., 2010; Shen and al., 2015)*. **Figure 8** includes all clinical trials and their results. Only 3 trials showed a positive effect. Lee and al. explain the significant numbers of negative trial by the lack of a patient selection based on the biomarker tested and the lack of consideration of the molecular subtype and stage of GC patients *(Lee and al., 2016)*.

The case of *H.pylori* treatment

In addition to the above anticancer protocols, patients are treated to eradicate *H. pylori* infection. Reviews on the subject in terms of benefit are very controversial in term of benefit. Patients are treated with proton pump inhibitors (PPI), which increases the acidity of the environment and eliminate the bacteria. For some patients, the significant increase in stomach acidity will result in

an increase in gastroesophagal reflux and suddenly an increased risk of developing cardia GC type or GOJ type *(Mihmanli and al., 2016)*.

5.2. Palliative treatment

Palliative treatment is applicable to unresectable and / or metastatic GC type. In this type of treatment, palliative gastrectomy may be done for symptomatic tumors (bleeding) *(Ouchi and al., 1998)*. It can be also combined with chemotherapy or chemoradiotherapy in order to improve patient's survival and quality of life. However patients who are not operated may receive chemotherapy in first line therapy (drug combinations used are equivalent to that of the curative treatment) *(Harvey and al., 2004)*.

Target	Trial	Type of Study/Line	Patient Selection Method	Regimen	Results (Primary Endpoint)	Reference
HER2	ToGa	Phase III/first	HER2 IHC	5-FU/capecitabine + cisplatin $\pm$ trastuzumab	Positive (OS)	Bang et al 2010[5]
HER2	LOGIC	Phase III/first	HER2 amplification	Lapatinib + XELOX / XELOX	Negative (OS)	Hecht et al[16]
HER2	TYTAN	Phase III/second	HER2 amplification	Paclitaxel + lapatinib vs. paclitaxel	Negative (OS)	Bang et al (2013)[17]
EGFR	EXPAND	Phase III/first	All comer	Cetuximab/XP vs. placebo/XP	Negative (OS)	Lordick et al[18]
EGFR	REAL-III	Phase III/first	All comer	Panitumumab/EOC vs. EOC	Negative (OS)	Waddell et al[19]
EGFR	Nimotuzumab	Phase II/second	All comer	Nimotuzumab/irinotecan vs. irinotecan	Negative	Kim et al[20]
VEGF	AVAGAST	Phase III/first	All comer	XP/bevacizumab vs. XP	Negative (OS)	Van Cutsem et al[21]
MET	RILOMET-1	Phase III/first	MET IHC	Rilotumumab/ECX vs. ECX	Negative (OS)	Iveson et al[22]
MET	METGastric	Phase III	MET IHC	Onartuzumab/FOLFOX vs. FOLFOX	Negative (OS)	Shah et al[23]
FGFR2	SHINE	R-Phase II/second	FGFR2 amplification	AZD4547/paclitaxel vs. paclitaxel	Negative (PFS)	Bang et al (2015)[24]
mTOR	GRANITE	Phase III/second or third	All comer	Everolimus vs. placebo	Negative (OS)	Ohtsu et al[25]
AKT	MK2206	Phase II/second	All comer	MK-2206	Response rate, 1%	Ramanathan et al[26]
ATM	Olaparib	R-Phase II/second	ATM IHC	Paclitaxel/olaparib vs. paclitaxel/placebo	Negative (PFS)	Bang et al (2015)[27]
VEGF	MEGA	R-Phase II/first	All comer	FOLFOX/aflibercept vs. FOLFOX	Negative (6-mo PFS)	Enzinger et al[28]
HER2	GATSBY	Phase II/III/ second	HER2 IHC	TDM1 vs. paclitaxel or docetaxel	Negative (OS)	Kang et al[29]
VEGFR-2	RAINBOW	Phase III/second	All comer	Paclitaxel/ramucirumab vs. paclitaxel/placebo	Positive (OS)	Wilke et al[30]
VEGFR-2	REGARD	Phase III/third	All comer	Ramucirumab vs. placebo	Positive (OS)	Fuchs et al[31]

Abbreviations: OS, overall survival; XP, capecitabine (Xeloda) and cisplatin; EOC, epirubicin, oxaliplatin, and capecitabine; IHC, immunohistochemistry; ECX, epirubicin, cisplatin, and capecitabine; PFS, progression-free survival.

Figure 8: Major Clinical Trials in Gastric Adenocarcinoma With Targeted Agents. *Lee and al., 2016*

II. THERAPY BASED ON ORGANOMETALLIC COMPOUNDS

## 1. Platinum compounds

1.1. Generality

As I have described previously, platinum-derived compounds are widely used in the curative or palliative treatment for GC. Many platinum derivatives have been synthesized, but only 3 are currently approved for worldwide diffusion and use, namely cisplatin, carboplatin and oxaliplatin. They are mainly used in combination with other anticancer drugs, such as antimetabolites 5-fluorouracil (5FU), and taxanes (placitaxel, docetaxel) for their synergistic anti-tumor activity *(Montagnani and al., 2011)*.
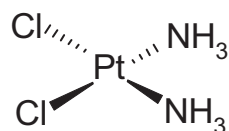
Cisplatin or cis-diammine dichloroplatinum (II) is currently one of the most used drugs in chemotherapy for its cytotoxic effect and low cost. This molecule was synthesized by M. Peyrone in 1844, but it was not until 1960 that its anti-tumor activity was described by M. Rosenberg. The first clinical phase I trial was realized in 1971. Soon after, cisplatin was approved by the FDA in 1978 exclusively for the treatment of testicular and ovarian cancer. Since then it has been approved for many cancers such as bladder, cervical, head and neck, stomach, small-cell and non-small cell lung cancer *(Dasari and al., 2014; Ali and al., 2013)*. Despite its role as standard in the treatment of cancer, there are two major limitations in its use: many *de novo* or acquired resistances have been described, and multi-toxicity was observed, including nephrotoxicity and emetic activity. This is why more than a hundred analogues were synthesized in the last fifty years in order to counteract the deleterious effects of cisplatin. Among these 2nd and 3rd generation analogs, only carboplatin (1989) and oxaliplatin (2002) have been approved for the treatment of many cancers *(Moncharmont and al., 2011).*
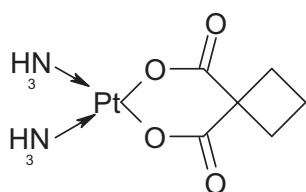
Carboplatin or cis-diammine (1,1-cyclobutanecarboxylato) platinum (II) demonstrated less side effects in comparison with cisplatin, especially the nephrotoxic activity. In terms of structure it differs from cisplatin by the presence of a bidentate dicarboxylate ligand instead of two chloride ligands observed for cisplatin. It has the ability to form the same interstrand DNA adduct as observed with cisplatin, which leads to significant resistance cross-reactivity with cisplatin *(Burger and al., 2011)*. It has a similar spectrum of efficacy to cisplatin but with a lower reactivity and a slower DNA binding kinetics. Four times

more carboplatin molecules are required to obtain an equivalent effect to cisplatin. Moreover, its significant higher cost leads to a limited use to the ovarian, lung and head and neck cancer. It is mostly described for its role in the tumor radiosensitivity. One of the major side effects of this treatment is its myelosuppressive activity that causes decreased production of blood cells and platelets in the bone marrow down to 10% of the usual production *(Carter and al., 1985)*.
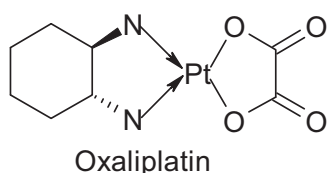
Oxaliplatin differs with cisplatin in its structure by the presence of a bulky diaminocyclohexane ligand (DACH). It has the ability to form the same DNA adducts as cisplatin but they are recognized by different repair proteins. The mismatch repair pathway (MMR) does not seem to be involved in the repair mechanisms following treatment with oxaliplatin in contrast with what is observed for cisplatin. Indeed, the cells presenting a loss of MMR are resistant to cisplatin, but not to oxaliplatin *(Scheeff and al., 1999; Meyerhardt and al., 2005)*. This change in the recognition of DNA damage allows a lower cross-reactivity with cisplatin. This is why it was the first drug to be approved to replace cisplatin. Originally, its use has been approved for colon cancer, and was then extended to many other cancers.

1.2. Mode of action of platinum compounds

Cisplatin, carboplatin and oxaliplatin are compounds of the alkylating family. They are composed of doubly charged platinum ion surrounded by four ligands. The amine ligands on the left side forming stronger interactions with the platinum ion, and the chloride ligands or carboxylate compounds on the right side forming leaving groups. This results in the capacity of the platinum ion to form bonds with DNA bases as described by *Dasari and al, 2014*.

1.2.1. Transport and biotransformation of Cisplatin

Cisplatin is active once inside the cell. Its transport into the cell is mostly passive, but it can also be active by the solute carrier importers (CRT1, AQP2, AQP9). Once inside the cytoplasm, cisplatin is hydrolyzed so the chlorides atoms will be replaced by mono or diaqua-molecules, which confer to cisplatin an electrophilic potential and allows it to interact with any nucleophiles such as the sulfhydryl group (-SH) on proteins and the nitrogen donor atom on the nucleic acid. Cisplatin binds to the N7 reactive center on purine residues, resulting in DNA damage, cell cycle arrest and apoptosis of the cell *(Cohen and al., 2001; Wilshaw, 1979; Siddik, 2003)*. The 1,2-intrastrand cross-

link of purine bases is the most notable change in DNA structure with 90% of d(GpG) adducts and 10% of d(ApG) adducts. 1,3-intrastrand d(GpXpG) and inter-strand adducts are also observed to play a role in the cytotoxicity of cisplatin (**Figure 9**) *(Crul and al., 2002; Boulikas,2007)*.
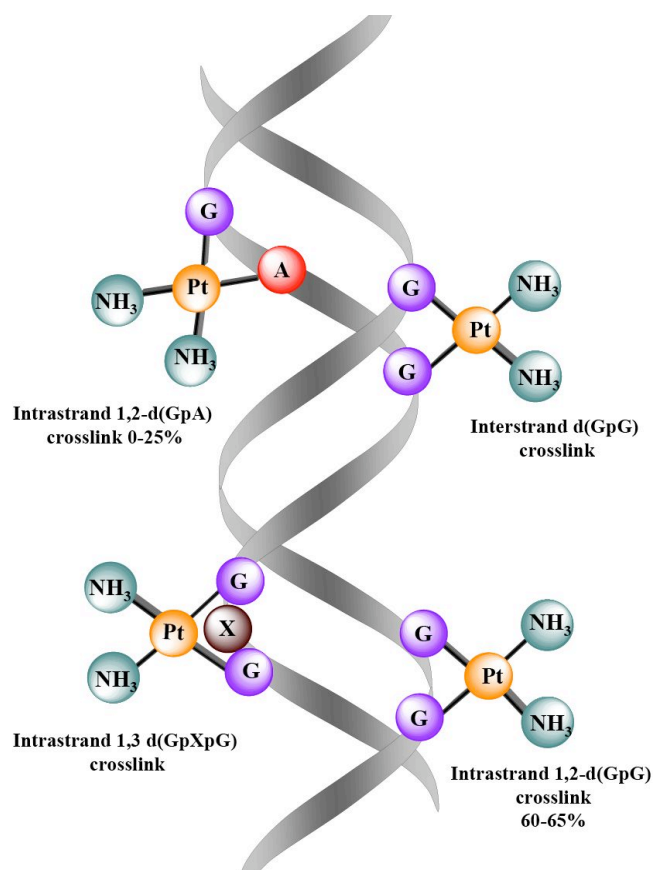
Figure 9: DNA adduct formation with cisplatin leaving two amino groups coordinated on the platinum atom, *Boulikas, 2007a*.

1.2.2.   Recognition of platinum-DNA adducts

The DNA-cisplatin adducts generate a distortion of the DNA double helix. This conformational change will allow exposure of the minor groove of DNA and thus the interaction with different nuclear proteins such as the HMG domain proteins, the repair proteins, and the transcription factors. Among them, the role of HMG box proteins has been mainly described in the recognition of the 1,2-intrastrand adducts *(Wang and al., 2005)*. HMG domain proteins are part of the large group of the non-histone chromosomal proteins. They have the ability to fix specific structures of DNA or chromatin with little or no sequence specificity. The HMG protein domains, HMG1, HMG2, UBF are known to recognize DNA-cisplatin adducts. They bind specifically to the cisplatin adducts and act as shield to prevent their repair. Thus, these proteins play an important role in the

sensitivity of cells to cisplatin *(Wozniak and al., 2002)*. To support this effect, overexpression of this recognition protein like HMG1 in breast cancer after pre-exposure of estrogen sensitizes cells to cisplatin *(He et al., 2000)*. Among these proteins, HMGB1 is known for its DNA-dependent activity. It has the ability to interact with the complex elements of the MMR, hMutSα and MLH2, to prevent DNA repair. It is also able to interact with some transcription factors such as p53, a tumor suppressor increasing its DNA-binding activity. Conversely, p53 itself is able to increase the affinity of HMGB1 for cisplatin adducts, which can prevent the damage repair by nucleosome excision repair pathway (NER) *(Wang and al., 2005; Wozniak and al., 2002)*.

1.2.3. DNA repair pathway

The distortion of DNA induced by the adduct formation of cisplatin-DNA will allow its recognition by different repair pathways constituted of more than 20 proteins. The pathways that have been the most involved in this context are the nucleotide excision repair (NER) and the path of mismatch repair (MMR) *(Shen and al., 2012)*.
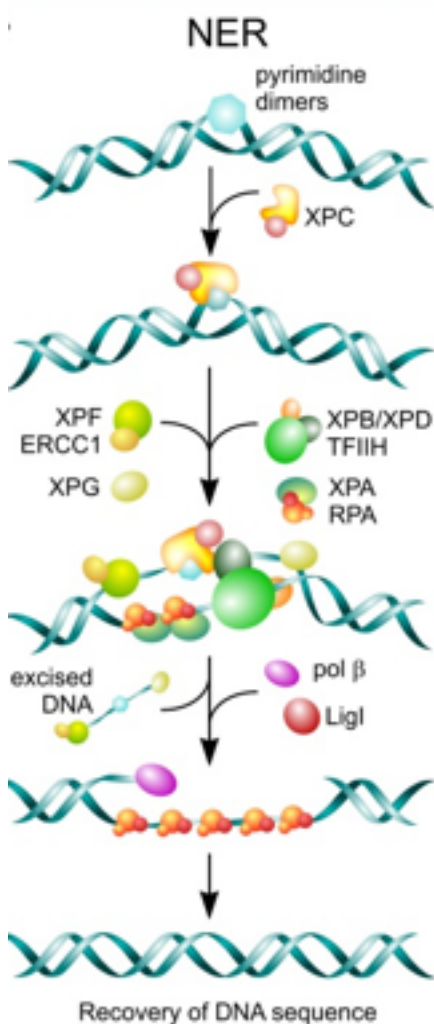
Figure 10: Nucleotide excision repair (NER) pathway. *Mladenov and al., 2011*

The NER pathway consists of several steps to correct the damage caused to DNA by cisplatin. The distortion of the DNA created by the cisplatin-DNA adduct will allow the attachment of many proteins of the NER *(Vasquez and al., 2002)*. After a lesion recognition step followed by their verification, they are incised / excised, which results in the removal of a 30 base pairs fragment of single-stranded DNA, DNA will then be resynthesized and ligated. There are two subtypes of NER, the transcription-coupled repair (TCR) and global genome repair (GGR). Briefly, the proteins involved in these steps are: the XPA-RPA complex enables the recognition / verification of cisplatin-DNA damage and allow the recruitment of the general transcription factor TFIIH compound of helicases (XPB and XPD). The action of helicases allows the binding of the endonucleases XPG and ERCC1 / XPF, which cut in 5' and 3' of the lesion and allow to remove the adducts *(Fink and al., 1996; Rosell and al., 2003;*

34

Seetharam and al., 2009). Cisplatin is one of the most used treatment for testicular cancer with high sensitivity. This is explained by a low capacity to remove adducts result in a decreased expression of the XPA and XPF factors. We can thus conclude that the NER is a key mechanism in the development of cisplatin resistance *(Welsh and al., 2004).*
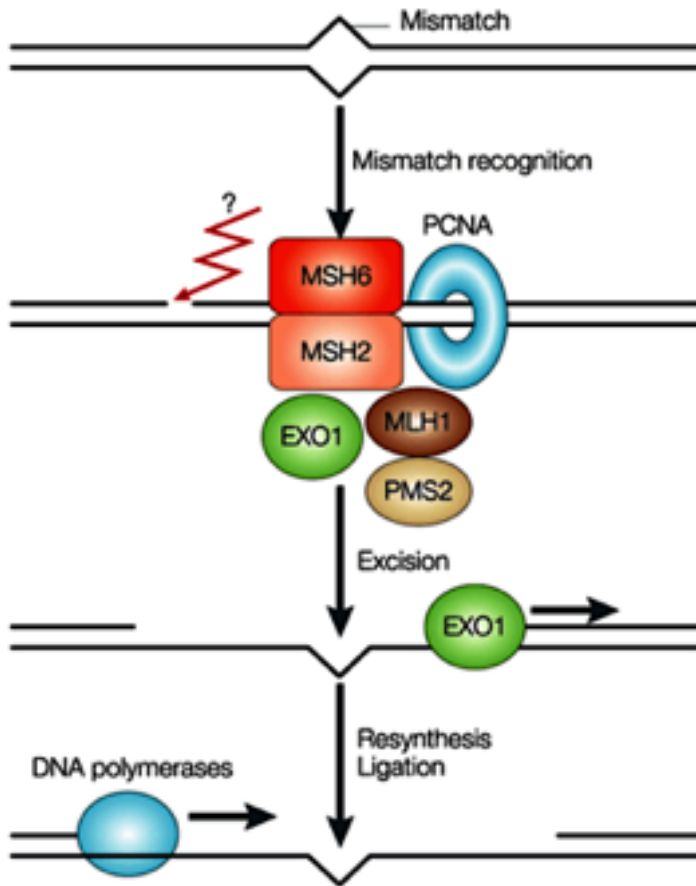
The MMR is a post-replicative DNA repair process involved in maintaining genome stability through the correction of single base mismatches and mismatches loop in newly replicated DNA

Figure 11: Representation of the MMR, *Martin and al., 2002*

Cisplatin-DNA adducts are recognized by the MutS complex (MSH2-MSH6) allowing the recruitment of MLH1, the post-meiotic segregation 2 (PSM2) and the exonuclease EXO1. After excision of the lesion, the proliferating cell nuclear antigen (PCNA) allows the attachment of the DNA polymerase to repair the lesion *(Manic and al., 2003)*. Instead of increasing cell viability, the MMR is known to be significant for cisplatin-mediated cytotoxicity *(Basu et al., 2010)*. Indeed the loss of function of the MMR, by decreased expression of MLH1 or MSH2, results in the development of cisplatin resistance in esophageal cancer *(Kishi et al., 2003)*. Two pathways are described to explain the role of MMR in the induction of apoptosis in response to cisplatin. First, the MMR can make 'futiles cycling' repairs, causing double-strand break of DNA and thus apoptosis. Moreover, MMR proteins are described for their role as a sensor of DNA damage, leading to the recruitment of ATM and ATR, two proteins involved in the checkpoint of the cell cycle, leading to apoptosis of the cell *(Jamieson and al., 1999; Bellacosa and al., 2001)*.

1.2.4. Transduction of DNA-damage signals

Cisplatin and p53 family protein

Part of cisplatin cytotoxicity is linked to p53- and p73-dependent apoptosis. The p53 family proteins are transcription factors that have a strong tumor suppressor activity. They are involved in many cellular processes such as DNA damage by regulating the cell cycle, cell death and DNA repair.

p53 is known to be mutated in 50% of cancers and inactivated in 60% of GC *(Hollstein and al., 1991)*. Upon genomic stress, such as cisplatin treatment, p53 acts in maintaining the genomic integrity by the transcriptional activity of its target genes such as *CDKN1A* (p21, cell cycle arrest) or *PMAIP1* (Noxa, pro-apoptotic).
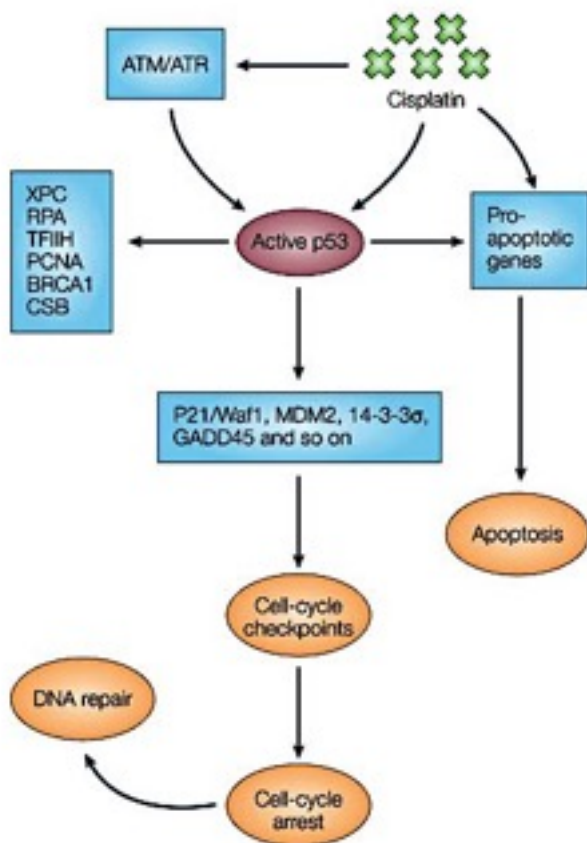
Figure 12: Transduction of DNA-damage signals: p53 and cisplatin. *Wang and Lippard, 2005*

In response to DNA damage (**Figure 12**), ATM phosphorylates and thus activates p53. p53 can then activate the transcription of its target genes, *CDKN1A* (p21), mouse dual minute 2 homolog (*MDM2*) or 45kD-growth arrest and DNA damage (*GADD45*). These targets will allow the cell cycle arrest and DNA repair. Moreover, p53 is described to interact with the proteins of the NER as XPC, TFIIH *(Adimoolamand al., 2002; 2003)*. Finally p53 can induce apoptosis of the cell by the transcriptional activation of pro-apoptotic genes such as *BBC3* (PUMA), a pro-apoptotic Bcl2-member, and it can bind directly on Bcl-xL, an anti-apoptotic protein, to counteract its activity. In addition, p53 induces the expression of PTEN, a tumor suppressor, which will enable the inhibition of the PI3K / Akt pathway involved in cisplatin resistance mechanisms increasing cell survival *(Basu and al., 2010)*. However, the cytotoxic activity of cisplatin is not

exclusively dependent on p53. Indeed, a breast cancer cell line highly sensitive to cisplatin shows an inactivation of p53 expression due to its sequestration by HPV-E6 virus *(Brabec and al., 2005)*.

p73 represents the second pathway involved in the cytotoxicity of cisplatin leading in cell apoptosis. Indeed, p73 is induced in response to cisplatin in many cancer cell lines and associated with the activity of c-Abl and MMR *(Gong and al., 1999)*. The MMR sensor activity will allow the activation of the c-Abl protein, a non-receptor tyrosine kinase, which is an upstream effector of several signaling pathways, as the Janus kinase (JNK), ERK / MAPK, p38 MAPK pathway. The phosphorylation and stabilization of p73 play an important role for apoptosis in response to cisplatine *(Siddik and al., 2003)*.

Oxidative stress and cisplatin

Under normal conditions, the cell controls the formation of reactive oxygen species (ROS) with a balance of ROS generation / elimination through various enzymes such as glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) *(Jungwirth and al., 2011)*. ROS have a role in cell signaling, including apoptosis, gene expression, and the activation of cell signaling cascades. Cisplatin, besides causing damage to DNA, also induces increased intracellular ROS accumulation. In addition, oxidative stress due to cisplatin is strongly linked to its nephrotoxic activity *(Saad and al., 2004)*. In fact, cisplatin directly targets the mitochondrion. Several studies have shown that cisplatin had a higher affinity for mitochondrial DNA than nuclear DNA. In addition, adducts removal efficiency in the mitochondria is lower than in the nucleus *(Kohno and al, 2015. Chen and Lesnefsky, 2011).* Moreover, Cullen and al. describe the ability of cisplatin to bind to one of the mitochondrial permeability transition (MPT) components, the voltage dependent anion chanel (VDAC), thus causing its opening *(Cullen and al . 2007)*. These two mechanisms resulted in increased ROS production and a decreased the antioxidant defenses, characterized by the loss of the mitochondrial proteins sulfhydryl groups. Moreover, inhibition of the mitochondrial respiration will induce a calcium efflux, a decrease in its mitochondrial membrane potential, the release of cytochrome c and thus cell apoptosis *(Florea and al., 2011; Marullo and al., 2013; Chirino and al., 2009).*

1.3. Side effect

One of the major limitations in the use of cisplatin is the significant development of side effects. In fact, cytotoxicity and cisplatin-dependent apoptosis do not target only the tumor cells but also normal cells. Among the most often listed side effects for cisplatin, there is in majority a nephrotoxic, neurotoxic, cardiotoxic and hepatotoxic activity *(Florea and al., 2011)*.

Nephrotoxicity

This is one of the most severe side effects reported for cisplatin. Cisplatin particularly accumulates in the kidney especially at the proximal tubular epithelial, being its main secretory pathway *(Köberle and al., 2010; Hartmann and al., 2003)*. Toxicity in the kidney could be explained by a decreased production of the L-carnitine, an enzyme implicated in the generation of metabolic energy and with an antioxidant role *(Chang B. and al., 2002; Al-Majed, 2007)*. Nephrotoxicity may be managed by a hyperhydration of patients with osmotic diuretic to increase body elimination of the drug.

Neurotoxicity

It affects 50% of patients with peripheral injury at the dorsal root ganglia. This results in the development of sensory neuropathy such as loss of sensation in the extremities for example. Neurotoxicity appears to be dose and time dependent, but the mechanisms involved are currently not elucidated *(Argyrriou, 2008, McWhinney and al., 2009)*.

Hepatotoxicity

Hepatotoxicity is the consequence, as nephrotoxicity, of oxidative stress caused by cisplatin. In fact, ROS cause a decrease in the antioxidant defenses such as the glutathione enzyme (GSH) and induce the increase of cytochrome P450 2E1, which leads to the toxicity of cisplatin in liver *(Liao and al., 2008)*.

Cardiotoxicity

Patients present electrocardiac changes, arrhythmias, myocarditis, cardiomyopathy and congestive heart failure. Cardiotoxicity could be a consequence of the oxidative stress and apoptosis increased. In fact, the lipid peroxidation of cardiac membranes following treatment causes the degeneration and necrosis of the cardiac muscle fibers cells *(El-Awady and al., 2011)*.

1.4. Resistance mechanisms

Besides the side effects, which in some cases require interruption of treatment, many resistance mechanisms have been described and are therefore another limit in the use of cisplatin. There are two types of resistance, intrinsic or innate resistance and those acquired during the treatment. Acquired resistance mechanisms may be due, to a decreased drug accumulation / increased drug efflux, modification of the intracellular drug distribution, an increased in the detoxification response (metallothionein, glutathione), an increased in DNA-damage repair (NER pathway), a

reduced apoptotic response, and to genetic and epigenetic modifications (miRNAs, HDAC…) *(Boulikas and al., 2007(b); Dasari and al., 2014,Siddik, 2003,Shen and al., 2012)*.

1.4.1. Transporters and reduced platinum accumulation

This is the most described mechanism of resistance to cisplatin. The decrease in cisplatin uptake and accumulation in the cells will result in the decrease in the adducts formation and thus in cisplatin cytotoxicity.

Copper Transporter 1 (CTR1) in Cisplatin uptake

As described by Howell et al., CTR1 is involved in the copper and cisplatin transport in the cell. Genetic knock-out of CTR1 is correlated with a decrease in cisplatin accumulation in the cell and thus in resistance to the treatment. Conversely, overexpression enables high accumulation of cisplatin and therefore sensitivity to treatment *(Howell and al., 2010)*.

ATP7A/B in Cisplatin efflux

They are analogues to the family of multidrug resistance-associated transporter (MDR) that are involved in the detoxification of many chemotherapeutic compounds *(Rabik and al., 2009)*. As CTR1, ATP7A and ATP7B, two copper-Transporting P-type ATPase-genes, are involved in the regulation of copper levels in the cell *(Safaei and al., 2008)*. ATP7B overexpression in cancer cell lines resistant to cisplatin is correlated to both a 9-fold increase in cisplatin resistance and 2 fold to copper. An increase in their expression in cancers such as ovarian cancer make them good clinical marker of chemosensitivity to cisplatin *(Safaei and al., 2005)*.

Transmembrane protein 205 (TMEM205)

It is a putative membrane transport involved in the decrease in cisplatin accumulation in the cell. An increase in its expression was shown in a cisplatin resistant cell line CP-r by Shen in 2010. In addition an analysis of its expression pattern in normal human tissues indicates a high level of expression in the liver, pancreas and adrenal glands. Also, TMEM205 could be used as a biomarker in the response to chemotherapy *(Shen and al., 2010)*.

1.4.2. Cisplatin inactivation by thiol containing molecules

The low concentration of chloride in the cell will allow cisplatin aquation activation. Cisplatin may be inactivated by glutathione (GSH), involved in the mechanisms of detoxification and protection against ROS *(Boulikas and al., 2007(b))*. This resistance mechanism is common in cases of chronic

exposure to cisplatin, which leads to an increase of GSH, which could be induced by Bcl-2 (anti-apoptotic protein). Cisplatin has the ability to react with molecules containing sulfide group, found in GSH. Overexpression of GSH will lead to the increase of cisplatin / GSH conjugate formation which will prevent the interaction of cisplatin with the DNA *(Lai and al., 1989)*.

1.4.3. Epigenetic changes and Cisplatin resistance

Several epigenetic pathways are involved in cisplatin chemosensitivity of the tumor. Thus epigenetic profile of many cell lines resistant to cisplatin allowed the identification of molecular modulators of these mechanisms *(Shen and al., 2012)*.

Changes in chromatin structure can be correlated with the development of resistance to treatment. Piwil2 is involved in resistance to cisplatin. It is a member of the PIWI / Argonaute family important in its involvement in the regulation of chromatin modifications, in RNA silencing and in the transcription of many genes. In fact, its increased expression, described in many cancers, results in chromatin condensation thus affecting the normal DNA repair *(Wang and al., 2011)*.

However, the aberrant promoter hypermethylation is a hallmark of a multidrug resistance. The analysis of epigenetic profile of cisplatin resistant cell lines has shown hypermethylation of many genes involved in the cytotoxic response to anticancer compounds such as p53 and p73 *(Wermann and al, 2010)*.

In addition, modification of histones plays an important role in maintaining the structure and function of the chromatin. Histone acetylation impacts positively the transcription and DNA repair. Overexpression of some histone deacetylase (HDAC), involved in chromatin condensation and thus the inhibition of transcription, has been demonstrated in many cancers. The treatment of a resistant ovarian cell line to cisplatin, the A2780 / CP70, with a methylation inhibitor (DAC) and a histone deacetylase inhibitor show an increased expression of MLH1 (MMR) and thus an increased sensitivity to cisplatin *(Steele and al., 2009)*.

Finally, microRNAs (***see part III.3***) play a crucial role in many biological processes such as in stress response, in apoptosis, and in proliferation, etc. In view of their function, it is not surprising that they have been described to have an impact in the sensitivity or resistance to cisplatin *(Bushati and al., 2007; Leung and al., 2010)*. Briefly some examples: overexpression of miR-214 leads to the

inhibition of its target gene PTEN, which promotes the survival of cells. Indeed, PTEN was previously described for its involvement in the p53-dependent cisplatin response *(Yang and al, 2008)*. Conversely, Sun and al. have shown that loss of expression of miR-200b and miR-15b reflects the establishment of the epithelial-mesenchymal transition (EMT) and therefore the acquisition of a tumor resistance to the treatment and an invasive phenotype *(Sun and al., 2012)*.

1.4.4. Gastric cancer and Cisplatin resistance

In GC, these mechanisms are not really understood, which reinforces the need to identify robust molecular markers. Some of them are described and are mostly implicated in activation of DNA repair. For example, overexpression or amplification of HER2 is implicated in the cisplatin resistance in GC cells, leading to the initiation of the epithelial-mesenchymal transition and correlated with an unfavorable outcome for patients *(Huang and al., 2013)*. Moreover, patients treated with cisplatin can exhibit an overexpression of ERCC1 and BRCA1, two enzymes implicated in the NER pathway, which are correlated with a worse prognosis. In the other hand, overexpression of the same enzyme, BRCA1, enhance sensitivity of the cells treated with docetaxel, which reinforces the complexity of the system *(Pietrantonio et al., 2013)*.

The identification of these markers will allow us firstly to classify the GC and adapt the treatment, and secondly, to develop targeted therapies, like for example the used of trastuzumab for HER2 overexpression, for patient with advanced GC with metastasis. This identification and progression in treatment means better knowledge of the mechanisms of action of therapies and tumor biology possibly leading to increased aggression. Despite genuine efforts to improve and better define the platinum-based treatments, there are still limitations to their usage. Indeed, they are only effective for a limited number of cancers, they cause severe side effects that require in some cases a treatment arrest, but especially more than 50% of tumors exhibit innate or acquired resistance mechanisms. In recent years, with a goal to counteract the adverse effects of the platinum compounds, different teams focused on the development of other organometallic compounds.

My PhD work contributed directly to the ongoing research and development of such anticancer compounds containing ruthenium.

2. Ruthenium compounds

2.1. Interest on ruthenium compounds

In order to counteract the limitations observed with platinum derivatives, there has been a growing interest for ruthenium compounds in recent years. Indeed, similar to cisplatin, ruthenium compounds exhibit cytotoxicity against cancer cells *in vitro* and *in vivo*, including cell lines resistant to cisplatin *(Bergamo and al., 2012)*. An analogous ligand exchange abilities to complex platinum is observed. On the other hand, in view of the different mechanisms of action from those of platinum derivatives, there is no cross-resistance observed with cisplatin and they also show a greater spectrum of activity. Finally, they induce a lower toxicity in healthy tissues due to their ability to use the physiological pathway of iron transport in the body via transferrin *(Antonarakis and al., 2010; Meggers, 2007; Bergamo and al., 2011; Galanski and al., 2003)*. In the 70s, Clarke et al. were the first to describe the ability of the ruthenium compound pentaammine (purine) ruthenium (III) to inhibit the DNA and protein synthesis in nasopharyngeal cancer *(Kelman, Clarke and al., 1977)*. Based on these results, a real attention was paid to these new organometallic compounds. Among those synthesized, only two have been tested in clinical trials, the KP1019 (phase I), and NAMI-A (phase II).

Ruthenium compounds have chemical properties making them good candidates for the development of new anti-cancer drugs. According to Bergamo et al., they have *(Bergamo and al., 2012)*:
- The possibility to occupy a high number of spatial positions with up 30 stereo isomers due to their expanded set of octahedral coordination geometry.
- A high number of potential accessory molecules that can be carried by the drug structure.
- An oxidation / reduction state that allows them to exist in the biological fluids in almost all the most important oxidation states from II to IV.

2.2. Ruthenium based molecules

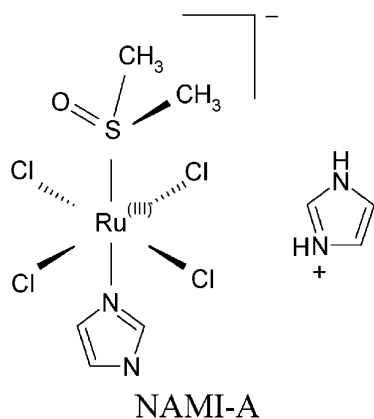The first compound described to have an anti-cancer activity is the ruthenium red which has the ability to inhibit calcium uptake into mitochondria thereby inducing apoptosis. Since then, many compounds based on Ruthenium (III) and (II) oxidation states were synthesized and tested for their cytotoxic effects *in vitro* and *in vivo*. In this part, I will describe the best characterized ones until now.

2.2.1. The Ru(III) compounds

Among the developed compounds of ruthenium (III), only the NAMI-A and KP1019 have been tested in clinical trial for their anti-cancer potential.

a. NAMI-A

NAMI-A, Imidazolium trans-imidazoledimethylsulfoxidetetrachlororuthenate was developed by Mestroni, Alessio and co-workers. This complex consists of a DMSO and imidazole molecules coordinated to the ruthenium (III). NAMI-A does not exhibit direct cytotoxic effect on tumor cells but it was described for its ability to inhibit the development of lung metastases and their growth once established and those in various types of solid tumors, including xenografts of human tumors. Associated with surgery of the primary tumor, a significant improvement in mouse lifespan in the Mca mammary carcinoma model is observed *(Gava and al., 2006; Alessio and al., 2004; Antonarakis and al., 2010)*. This effect is independent of the stage and pre-treatment of the tumor. It is the first derivative of ruthenium to have been tested in clinical trials in 1999 and reported in 2004 for its anti-metastatic properties *(Rademaker-Lakkai and al., 2004)*. Among the 24 patients included in the study with refractory solid tumors to standard chemotherapies, all showed good tolerability of the drug and 20 attenuated metastatic development. In addition, *in vitro* NAMI-A has been shown to act synergistically with other standard chemotherapies such as cisplatin. Thus a clinical trial to test the combination of NAMI-A with Gemcitabine for the treatment of non small cell lung carcinoma (NSCLC) was performed *(Bergamo and al., 2012)*.

NAMI-A anti-metastatic activity

NAMI-A has the ability to inhibit the metastatic tumor growth by modulating the different steps in the process: detachment, invasion / migration, re-attachment on a distant organ. Indeed it has been shown in the murine model of mammary carcinoma Mca that when tumors pretreated with NAMI-A were reimplantated, mice did not exhibit lung metastases formation. These experiments demonstrate the ability of this compound to purge the heterogeneous population of primary tumor cells for the elimination of those with metastasis ability *(Gava and al., 2006; Alessio and al., 2004; Antonarakis and al., 2010)*. NAMI-A induces the cytoskeleton rearrangement by interfering with the polymerization / depolymerization process of the F-actin and by modulating the regulation of TGFB1 in fibroblasts and tumor cells. These two actions will help to cause a fibrotic

reaction leading to stronger adhesion of the tumor capsule. Its ability to interact with integrins especially integrin B1 reduces cell migration. Indeed, integrin have an important role in the control of cell motility by regulating the cytoskeletal structure. The activity of metalloproteinase (MMP), regulator of extracellular matrix (ECM), depends on an intact cytoskeleton. In this context, there is a decrease in their activity especially for MMP2 and MMP9 in the presence of NAMI-A, leading to an increase of the ECM around the vascularisation of the tumor and a decrease in tumor cell invasion *(Vacca and al., 2002; Sava and al., 2003)*. Neo-angiogenesis is a crucial mechanism in the development of metastasis allowing them to reach distant organs. NAMI-A has the ability to completely inhibit this process set up by the vascular endothelial growth factor (VEGF) by its capacity to interact with the integrins. Their activation will allow the modulation of PKC activity, the dephosphorylation of ERK and the inhibition of the transcription of c-myc leading to the endothelial cells death *(Bergamo and al., 2007)*. NAMI-A is able to inhibit the development of metastases, but also the growth of those already established. Indeed in the animal model Mca mammary carcinoma, decreased of the metastasis number of metastases and their proliferation was observed. This effect is explained by a cell cycle arrest in G2 / M phase leading to a less invasive phenotype for the cells.

b. KP1019
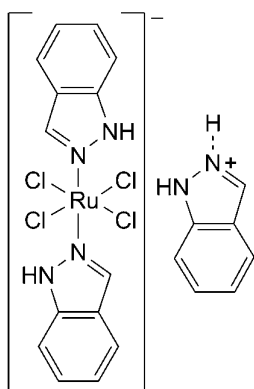
KP1019

KP1019 or Imidazolium trans-((tetrachloride)bis(1H-indazole) ruthenate(III)) was developed by the group of BK Keppler *(Hartinger and al., 2006)*. It consists of four equatorial chloride and two indazole heterocycles coordinated to the metal through a nitrogen atom. This is the second ruthenium compound to reach clinical trial (2000) in view of its direct cytotoxic activity *in vitro* against a cisplatin resistant colon cancer cell line. KP1019 is generally described as a prodrug active once into the cell because of the « reduction by oxidation » reaction *(Pizarro and al., 2009)*.

KP1019 cytotoxic activity

KP1019 has the ability to interact with serum proteins such as transferrin and thus to mimic the physiological pathway of the iron transport *(Messori and al., 1996)*. Indeed the transferrin proteins are crucial for the proliferative tumor that requires more iron than other tissues and thus increases the expression of transferrin receptor (CD71). This allows the KP1019 active transport into the cell

but also a specific recognition of tumor cells rather than healthy tissue *(Spreckelmeyer and al., 2014)*. KP1019 becomes active once inside the cell by the reduction of the ruthenium (III) to ruthenium (II) oxidation state according to the hypothesis of the «reduction by oxidation». Indeed, tumor cells are characterized by a low oxygen concentration and low pH, a consequence of the hypoxic status of the tumor. This allows the change in redox potential of the metal and the activation of the drug. Its direct cytotoxic activity is mediated through its ability to interfere with the electron transportation chain, which causes the depolymerization of the mitochondrial membranes. Apoptosis is then induced by a decrease in the expression of the anti-apoptotic factor Bcl-2 and the induction of Caspase 3 *(Clarke, 2002)*. KP1019 is able to interact with DNA equivalently to cisplatin but with a lower efficiency (15 times less). This causes double strands break in DNA but they are not sufficient to induce cell death directly *(Bergamo and al., 2012)*.

In 2005, Kepler and Heffeter described a mechanism of resistance for KP1019 involving the ABC transporters. Resistance to treatment is observed in numerous cell lines which overexpressed some receptors like the P-glycoprotein, which causes a reduction of the compound accumulation in the cell and thus halved its activity *(Heffeter and al., 2005; Trondl and al., 2014)*.

2.2.2. The Ru(II)-arene ligand compounds

Studies into the mode of action of KP1019 has highlighted the concept of pro-drugs and more precisely its activated form Ru (II). This has led researchers to synthesize new compound such as the ruthenium (II) arena-type complexes, which shows significant anti-proliferative effects *in vivo* and *in vitro* in cisplatin resistant cell lines. The arene ligands can stabilize the structure of the Ru (II) and provides a hydrophobic surface to the complex, which facilitates its transport across the membrane and the interaction with various biomolecules.

a. RM175
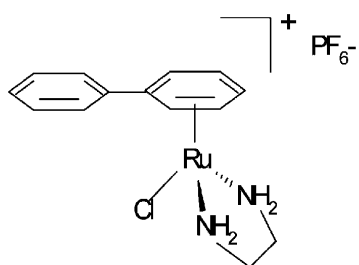
The RM175 is a biphenyl ethylenediamine chlorido Ru (II) complex developed by Peter Sadler. The advantage of this compound is its bifunctional effect on DNA, allowing its interaction with DNA by conventional N-bonding, and its intercalation between the bases of DNA through its biphenyl ligand *(Chen and al., 2002; Chen and al., 2003)*. The resulting DNA lesions are repaired by another mechanism than the NER pathway preventing cross-resistance with the platinum compounds. This compound has an in vitro cytotoxicity similar or higher than the carboplatin in the human colon and ovarian cancer cell

lines *(Bergamo and al., 2007)*. Hayward showed that in the HCT116 colorectal cancer cell line, RM175 triggered cell apoptosis by inducing the accumulation of p53 protein and its target gene *CDKN1A* (p21), leading to the cell cycle arrest in G1 / G2 phase. Cell apoptosis is mediated by p53 and the pro-apoptotic factor Bax *(Hayward and al., 2005)*. Interestingly, *in vivo* treatment of the ovarian cancer cell line A2780 with RM175 shows a significant growth delay of the tumors *(Aird and al., 2002).*

b. RAPTA complexes
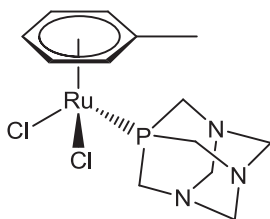
RAPTA complexes were developed by Dyson and have a [PTA] ligand. This ligand gives them the capacity to a pH-dependent activation enabling high selectivity for hypoxic cells at low pH. This compound was inspired by the RM175 of Peter Sadler with a change of the ethylenediamine ligand by the PTA ligand. There are several RAPTA analogs including RAPTA-T and RAPTA-C, which I will describe now.

RAPTA-T has an anti-metastatic activity similar to NAMI-A as has been demonstrated *in vitro* in the highly invasive MDA-MB-231 ovarian cancer cell line. *In vivo*, the two compounds provide growth inhibition of lung metastases with only a slight effect on the primary tumor. Their relatively simple structures allow them to be involved in intracellular and extracellular processes. Each compound has the ability to interact with DNA like cisplatin but they are much less effective. Furthermore the lesions are mostly repaired which proves that their cytotoxic activity is not DNA dependent *(Scolaro and al., 2005; Bergamo and al., 2008).*

RAPTA-T and RAPTA-C mode of action

Similarly these compounds trigger a cell cycle arrest in G2 / M phase followed by cell apoptosis. Indeed, RAPTA induce the accumulation of p53 and its target gene *CDKN1A* (p21) and a decrease in Cyclin E leading to the mitochondrial apoptosis with the release of the cytochrome C and the activation of the pro-caspase 9. Furthermore, JNK would be critical in the inhibition of cell growth by RAPTA-C *(Chatterjee and al., 2008)*.

c. DW 1/2

Meggers's team used a different strategy for the development and synthesis of new ruhtenium complexes. Indeed, he had used metal complexes as a structurally inert scaffolds for enzyme inhibitors based on the structure of staurosporine, resulting in compounds DW1 / 2. The ATP-competitive indolocarbazole alkaloid staurosporine, potent inhibitor of various kinases, was used as a lead structure to further develop the compound by replacing the carbohydrate unit of

staurosponine with ruthenium fragments. The interest to develop compounds that target DNA has decreased over the years in view of the lack of specificity of the activity of the compounds and thus the appearance of severe side effects. Moreover, with the peak of molecular biology, researchers are working to identify markers of cancer progression to target them specifically, especially those involved in the development of chemoresistance. In this context, targeted therapies such as the monoclonal antibodies, bevacizumab (anti-VEGF) and trastuzumab (anti-HER2) have already been developed.
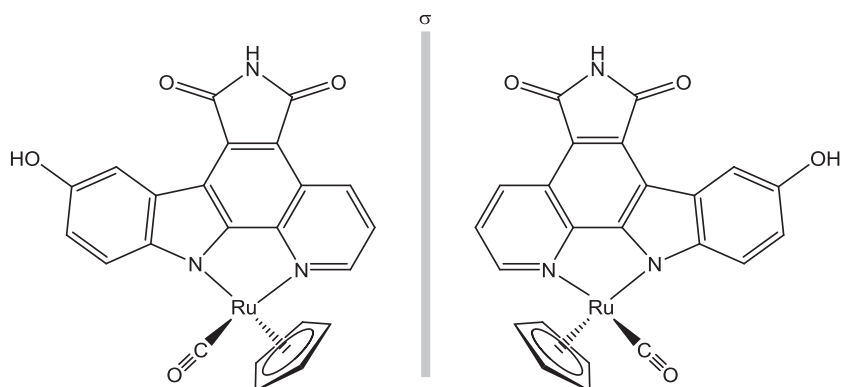
Among these compounds, DW1 / 2 racemic mixture specifically target the glycogen synthase kinase 3B (GSK3B). This protein kinase exhibits aberrant activity in many pathologies (Alzheimer's, Parkinson's desease) and in solid tumors (colon, liver, pancreas). It also plays a role in the generation of cancer stem cells. GSK3B is a key modulator of glycogen synthesis and can regulate other biological processes such as cell cycle, apoptosis by inhibiting p53 and thus the expression of its target genes (p21, *GADD45*) or cell motility. Smalley's team has shown the ability of DW1 / 2 to induce apoptosis *in vitro* in a model of melanoma. GSK3B specific inhibition allows p53 accumulation by inhibiting the expression of MDM2 and MDM4, two negative regulators of p53. p53 will allow the establishment of apoptosis by the intrinsic mitochondrial pathway *(Smalley and al., 2007).*

d. Ruthenium derived compounds (RDC)

The RDCs are developed by the team of C. Gaiddon and M. Pfeffer. These are cycloruthenated complex. Most of the ruthenium-containing compounds described so far have ligands that are bound to the metal via a heteroatom (N, O, S). In order to improve the stability of ruthenium complexes, the groups of Dr. Pfeffer and of Dr. Gaiddon have in an interdisciplinary effort generated and tested the biological activity of over 100 new ruthenium-based complexes in which the ligand is bound to the metal via strong covalent bonds C-M. This C-M bond is thought to

improve the stability of the molecule, confer a specific range of redox potential and allow them to be efficient mediators of electron transfer to or from oxidized or reduced active sites of redox enzymes, thereby affecting their activity *(Ryabov and al., 2001; Fetter and al., 2011)*. Moreover, these ligands may additionally confer useful physical properties to the organometallic moiety (such as fluorescence), thus enabling the metal and ligand to be traced in the cell in vivo.
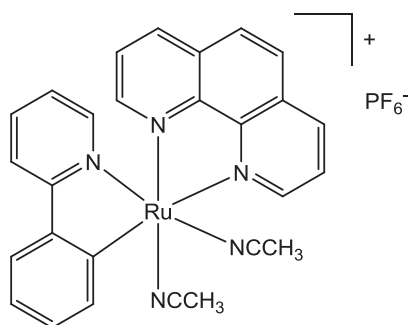
One of the compounds of this family, RDC11 was particularly studied due to a set of physical, chemical and interesting biological properties. It has cytotoxic activity generally stronger than platinum derivatives (IC50 ± 2uM) and is active on platinum resistant cell lines (Caco2, HCT116 p53 -/-). In vivo, it reduces tumor growth in several syngenic (melanoma, F10B16) and xenografts (U87 glioblastoma and A2780 ovarian cancer cell lines) tumor models. It has also lower kidney and neuronal chronic toxicity than cisplatin. In addition, the RDC11 is less sensitive to the mechanisms of cisplatin resistance as described for the over-expression of the ATP7B transporter, which does not diminish the accumulation of the compound into the cell. Studies on RDC11 mode of action have shown that this compound can induce cell death or growth arrest, by DNA dependent and independent mechanisms *(Meng and al., 2009; Gaiddon and al., 2005).*

RDC11 mode of action

Our laboratory showed that RDC11 was able to induce the expression of p53 and p73, as observed in response with cisplatin. Apoptosis of tumor cells is maintained in p53 -/- cell lines in response to the treatment. Furthermore, overexpression of dominant negative p73, ΔNp73, partially inhibits the ability of RDC11 to induce apoptosis. These experiments show that RDC11 has a p53 dependent and independent cytotoxic activity. The RDC11 has also been described for its ability to induce the oxidative stress by its accumulation in the mitochondria thereby generating ROS production and the establishment of intrinsic mitochondrial apoptotic pathway. On the other hand, the RDC11 is also capable to accumulate in the endoplasmic reticulum, thus involving the stress of the endoplasmic reticulum pathway (**Figure 13**) *(Gaiddon and al., 2005; Meng and al., 2009; Vidimar and al., 2012; Klajner and al., 2014).*
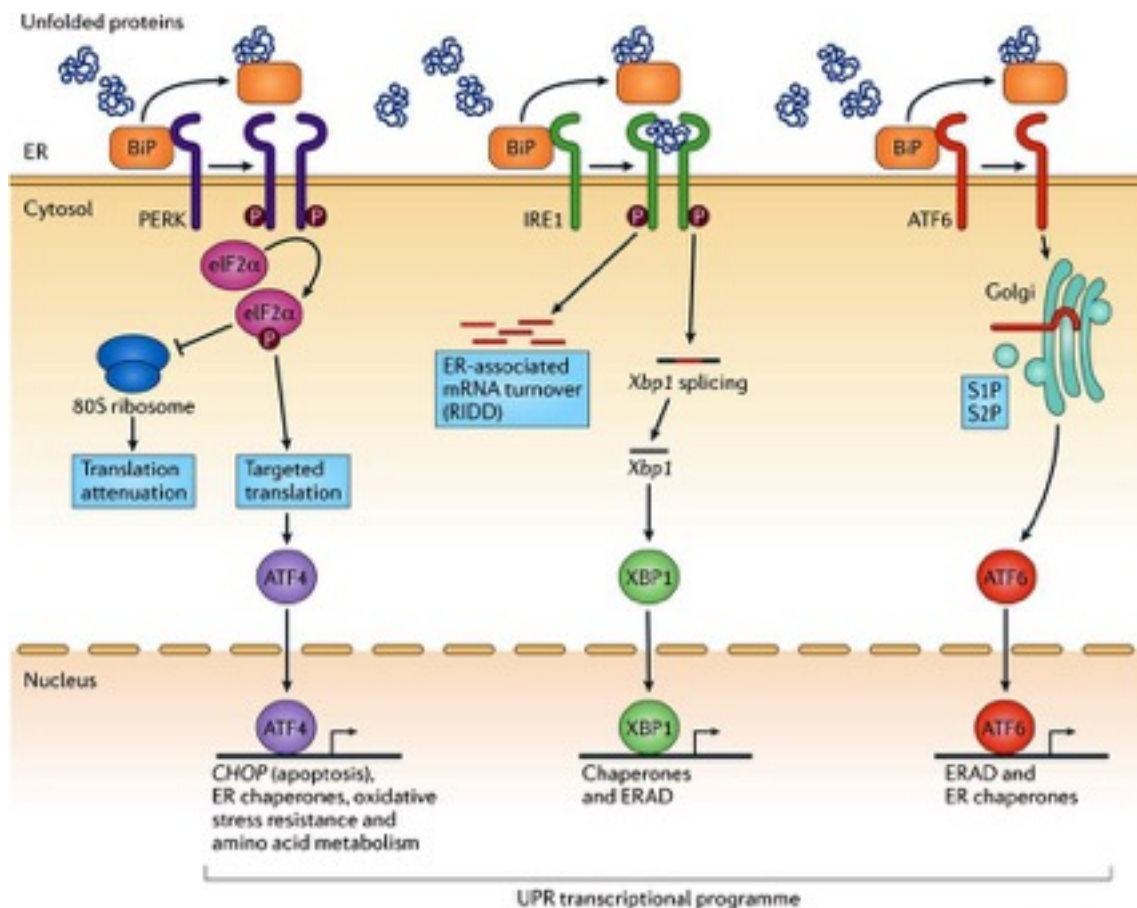
Figure 13: Cellular responses to endoplasmic reticulum stress. *Celli and Tsollis, 2014*

The endoplasmic reticulum is important in protein synthesis and is involved in glucose metabolism as well as calcium homeostasis. Disruption of cellular homeostasis by tumor processes, such as oxidation, malformed proteins and glucose deprivation, causes the activation of the endoplasmic reticulum stress pathway (UPR, Unfolded protein response), which allows either to restore homeostasis or cell death. The UPR pathway is characterized by the activation of three proteins: the protein kinase RNA-like ER kinase (PERK), the inositol-Requiring protein 1a (IRE1a), and the activating transcription factor 6 (ATF6). PERK activation generally induces the decrease of protein synthesis by the phosphorylation of EIF2a. EIF2a allows activation of ATF4 protein responsible for activating the ISR (integrated stress response), which aims to restore cellular homeostasis by controlling redox levels and cellular energy. However, PERK protein may also have pro-apoptotic effects by activating Ero1 protein, which is a type of redox protein PDI (protein disulfideisomerase) controlling the opening or closure of disulfide bonds between cysteine residues of certain proteins. The ATF6 protein is responsible for the synthesis of CHOP and XBP1 transcription factor. CHOP induces the expression of pro-apoptotic genes such as CHAC1 and TRB3. XBP1 is involved in degradation of the unfolded proteins and lipids. Serine threonine IRE1 kinase causes the splicing of XBP1 leading to the expression of an active transcription factor (XBP1s) that up-regulates a subset

of UPR target genes related to protein folding, ER-associated protein degradation (ERAD), protein quality control, and organelle biogenesis *(Brown and al., 2012; Oyadomari and al., 2004; Dalton and al., 2013; Yada and al., 2014).*

Meng and al showed that the RDC induced the expression of certain key modulators of this pathway such as XBP1, PDI and CHOP. CHOP induces the expression of two pro-apototic target genes, TRB3 and CHAC1. In addition, they showed that the cytotoxic activity of RDC11 was CHOP-dependent by inhibiting its expression by RNAi. The ability of RDC11 to induce multiple and independent stress response pathways represents an interesting property for anticancer drugs that might allow a broader spectrum of action and maybe explain why RDCs are less sensitive toward cisplatin resistance mechanism *(Meng and al., 2009)*.

III. EPIGENETIC MODIFICATIONS IN GASTRIC CANCER

1. The various epigenetic processes

1.1. Generality

Epigenetic was originally defined by CH Waddington in the context of the embryogenesis as: « the causal interactions between genes and their products, which bring the phenotype into being ». The establishment of the epigenetic program is crucial during development, and its stability is essential for maintaining the functions of each cell type in the life of an organism. Commonly, it is therefore referred to as « the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence » *(Sharma and al., 2010)*.

The epigenetic modifications will have as consequences to modify the chromatin structure, making it compact or accessible to the transcriptional machinery, and to influence gene expression. Epigenetic modifications concern the DNA methylation, the covalent and non-covalent histone modifications and the non-coding RNAs. These different pathways are interconnected and represent the guardians of the cell identity *(Kanwal and al., 2012)*.

A failure of the proper maintenance of heritable epigenetic marks can result in inappropriate activation or inhibition of various signaling pathways leading to the development of different pathology *(Egger and al., 2004; Jones and al., 2002)*. Cancers such as GC, are defined as heterogeneous and multifactorial diseases, consequences of both genetic and epigenetic changes. The study of the genetic causes of cancer (mutations, amplifications, loss of chromosome material…) has long been at the forefront in research. The new high-throughput sequencing techniques have allowed molecular analysis of cancers (**see Molecular characterization of GC**), leading to the emergence of new molecular actors implicated in epigenetic modifications in carcinogenesis *(Jones and al., 2005)*. They show the ability to modulate the chromatin structure, and so control the expression of genes. These new studies have revealed the important role of epigenetic changes in the initiation and progression of cancers. Moreover, unlike the genetic changes, epigenetic modifications are dynamic and reversible. Characterization of specific inhibitors of certain epigenetic effectors opened the possibility of new therapeutic approaches which seems to be really promising, with some molecules already approved by the FDA and other in clinical trials *(Yoo and al., 2006)*.

1.2. DNA Methylation

DNA methylation plays an important role in the maintenance of genome integrity, in genomic imprinting, transcriptional regulation and in developmental processes *(Wu and al., 2010)*. It enables a stable gene silencing which will have consequences for the regulation of gene expression, chromatin architecture, modification of histones and non-histone proteins. DNA methylation occurs preferentially in regions rich in dinucleotides of cytosine (C) and guanine (G) distributed unevenly in the genome: the CpG islands *(Bird, 2002; Takai and al., 2002)*. These islands are under-represented in the genome and located at the 5' end of 60% of genes in the promoter or the first exon *(Wang Y. and al., 2004)*. Furthermore, methylation of the non-transcribed regions is also observed. These genomic regions have repeated sequences at the centromeres, the gene body and the retrotransposon elements (**Figure 14**) *(Suzuki and al., 2008; Robertson, 2005; Portela and al., 2010).*

a).
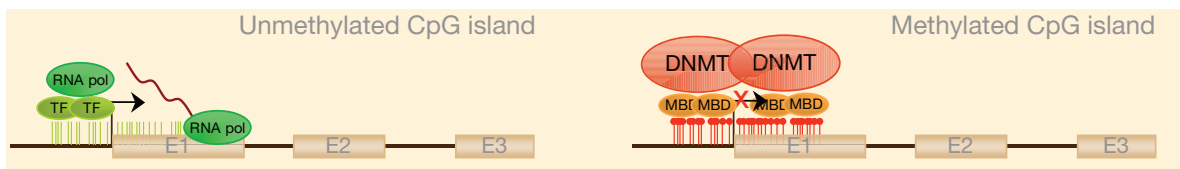
b).

c).
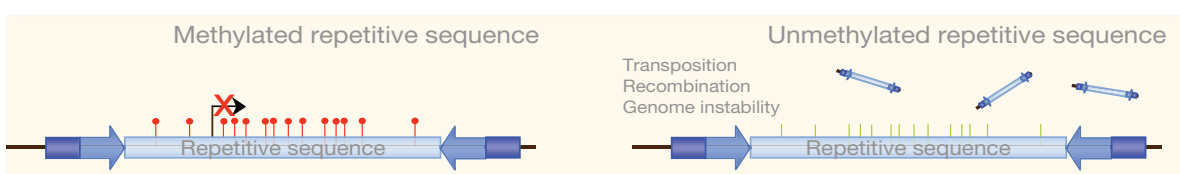
Figure 14: DNA methylation processes a). CpG island at promoters of genes are normally unmethylated, allowing transcription. Aberrant hypermethylation leads to transcriptionnal inactivation. b). Methylation at the gene body facilitating transcription by preventing spurious transcriptions initiations. c). Repetitive sequences appear to be hypermethylated, preventing chromosomal instability, translocations and gene disruption through the retrotransposons element. *Portela and al., 2010*

Methylation is an early event during the development through the action of three enzymes, the DNA methyltransferases (DNMT): DNMT1, Dnmt3a and DNMT3b. These enzymes allow the transfer of a methyl group from the donor molecule S-adenosyl-L-methionine (SAM) to the carbon 5 of cytosine *(Friedman and al., 2009)*. DNMT1 is involved in the maintenance of the epigenetic information in the next generation. It has the ability to methylate hemimethylated DNA during the S phase of the cell cycle. Conversely, DNMT3a and DNMT3b enzymes allow *de novo* methylation of the DNA independently of the replication. These enzymes can act on unmethylated or hemimethylated DNA. They are responsible for changes in epigenetic information allowing the silencing of certain genes of interest. DNMT3a/3b have a critical role in the initiation and progression of cancer and are frequently overexpressed in cancers, unlike DNMT1 *(Sachan and al., 2015)*.

Methylated CpG islands are recognized by a conserved protein motif of about 70 amino acids, called Methyl-CpG-binding domain (MDB). This domain is found in a protein family whose main members are MeCP2, MBD1, MBD2, MDB3 and MDB4 *(Filion and al., 2004*). However, Kaiso a transcription factor, containing no MBD but a zinc finger structure within a range BTB / POZ (Broad complex, Tramtrack and bric a brac / poxvirus and zinc finger) was also described for its ability to bind methylated CpG *(Deltour and al., 2005)*. These factors have the ability to recruit different complex leading to the histone post-translational modifications called the covalent histone modifications (such as methylation and deacetylation), resulting in a change of the transcriptional gene expression *(Nan and al., 1998)*.

1.3. Histone modifications

Chromatin Structure

The chromatin is formed of several repeated nucleosome units, which consists of 146pb DNA wrapped twice around an octamer of four core histone proteins, the nucleosomal histones: H3-H4-H2A-H2B *(Kornberg and al., 1974 ; Kornberg et al., 1995)*. Nucleosomes are spaced with 50bp of naked DNA and the histone H1, an internucleosomal histone or linker. They represent the most abundant proteins in chromatin and exhibit a strong conservation of their sequence except for H1. The nucleosomes are assembled into complex structures (spirals, solenoid, helix) to form the chromosome. The level of compaction involves different non-histone proteins including the topoisomerase II. Two types of chromatin are observed: **Euchromatin** (relaxed and transcribed structure) and **Heterochromatin** (condensed and inaccessible to the transcriptional machinery like in the centromere and telomere). *(Felsenfeld and al., 2003)*

Non-covalent histone modifications

The non-covalent histone modifications include the change in the nucleosome positioning and the replacement of the canonical histones by histone variants. As described above, the nucleosomes allow the packaging of DNA in the cell and so the regulation of gene expression through the alteration of the regulatory DNA sequences accessibility for the transcription factors *(Jiong and al., 2005)*. Genome wide studies of nucleosome mapping have shown the precise positioning of nucleosomes in the vicinity of gene promoters, specifically at the transcription start site (TSS), which influences the initiation of transcription *(Mayrich and al., 2008)*. The nucleosome free region (NFR) is present in 5' and 3' end of genes and allows the assembly / disassembly of the transcriptional machinery. The modulation of the NFR is regulated by the ATP-dependent chromatin-remodeling complexes which modify the accessibility of DNA *(Smith and al., 2005)*. These chromatin remodeling complexes are classified into 4 groups: SWI / SNF, ISWI, CHD and INO80 *(Roberts and al., 2004; Ho and al., 2010)*. They allow the binding or exclusion of nucleosomes. Thus, they will have a role in many cellular processes such as the regulation of gene expression (p21 regulation by SWI / SNF) or the alternative splicing modulation, DNA repair, telomere regulation, chromosome segregation and DNA replication *(Villar-Garea and al., 2003)*. Moreover, SWR1, a member of the INO80 family, is the only one to present the ability to restructure the nucleosome by removal of the H2A-H2B dimers, replacing it with the H2B-H2A.Z dimers, a histone variant *(Bachman and al., 2003)*. The interaction of the nucleosome remodeling machinery with the DNA methylation and the covalent histone modifications plays a crucial role in the overall establishment of gene expression and chromatin architecture pattern. In addition to the alterations in the nucleosomes positioning, incorporation of histone variants can be observed. In contrast to canonical histones whose synthesis and incorporation is coupled to the DNA replication in S phase, the histone variants are present all along the cell cycle. They will have a role in nucleosome positioning and gene expression. For example, histone variant H3.3 and H2A.Z were found at the active gene promoters *(Santenard and al., 2009)*. H2A.Z helps to protect genes against the DNA methylation *(Zilberman and al., 2008)*. In embryonic stem cells, H2A.Z is colocalizes with the bivalent domain, allowing the maintenance of genes involved in development. Histone variants, such as canonical histones can undergo covalent modifications *(Creyghton and al., 2008)*.

Covalent histone modifications

Histones are composed of a globular C-terminal end and an unstructured N-terminal tail. Post-translational modifications of histones take place on their tail, such as methylation, acetylation, ubiquitinylation, sumoylation and phosphorylation. These changes allow the regulation of key processes such as transcription, replication and DNA repair. They will induce a change in the

chromatin accessibility for the non-histone effector proteins and therefore activation or repression of the transcription as a function of epigenetic marks. The different combinations of epigenetic marks observed (acetylation+methylation for example) form an epigenetic code and are interdependent *(Jenuwein and al., 2001)*.

The covalent histone modifications currently described are the acetylation and methylation. The other histone modifications (phosphorylation, ubiquitinylation and sumoylation) will not be mentioned here. Acetylation of some histone lysine residues will induce a chromatin state called "open" and therefore transcriptionally active, whereas the effect of methylation is dependent on the residue involved (lysine or arginine) and on the number of methylated residues. For example, trimethylation of histone H3 on lysine 4 (H3K4m3) will activate the promoter of the gene concerned. Conversely, the epigenetic mark H3K9m3 and H3K27m3 will repress promoter activity. Interestingly, the embryonic stem cells (ES cells) have a bivalent domain allowing them to ensure their pluriopotency and self-renewal (*Kouzarides, 2007)*. This bivalent domain exhibits an active mark for transcription, H3K9m3 and one inhibitory for the transcription, H3K27m3, in gene promoter involved in the development *(Bernstein and al., 2006)*. Both marks will be handled by two critical regulators:

- The **polycomb,** which catalyzes H3K27m3 to allow the inhibition of cell fate specific genes and therefore maintain the pluripotency of ES cells.
- The **trithorax group,** which catalyzes H3K4m3 important to maintain active chromatin during development.

These mechanisms allow phenotypic plasticity. Thereafter, the differentiated cells will lose their bivalence and acquire a more rigid chromatin, allowing the maintenance of cellular identity *(Ringrose and al., 2007).*

Histone modifications are controlled dynamically by different enzymes. The histone acetyltransferase (HAT) and histone methyltransferase (HMT) respectively allow the addition of a acetyl and methyl groups *(Haberland and al., 2009; Shi, 2007)*. Histone deacetylase (HDAC, see section below) and histone demethylase (HDM) induced loss of this group. These enzymes interact with each other as well as other DNA regulatory mechanisms for modulating the status of chromatin and thus transcription. Two main functions are advanced for the covalent histone modifications *(Parra, 2015)*:

- Modify the electrostatic balance between the histones and thereby modulate the chromatin condensation status, allowing access to protein factors.
- Be the target of nuclear factors specifically recognizing these epigenetic marks.

Epigenetic signature is observed in the context of cancer. Indeed, a general DNA hypomethylation is observed with a local hypermethylation *(Sachan and al., 2015)*. Hypomethylation relates to oncogenes coding regions, but also to repeated sequence regions critical in maintaining stability of chromosomes, inactivation of transposable elements and genomic imprinting *(Watt et al. 2000)* . Inversely, the local hypermethylation causes transcriptional repression of genes with tumor suppressor role *(Esteller 2002; 2007; 2008)*. Indeed, the DNA methylation and histone modifications interact together to determine gene expression, chromatin organization and cellular identity. The HMT enzymes allow the recruitment of the DNMT at specific genomic loci to stably silent genes. The HMT and HDM also have the ability to influence DNA methylation levels by regulating the DNMT protein stability. Furthermore, the DNMT have the ability to recruit HDACs and MBP thus allowing inhibition of transcription and chromatin compaction *(Cedar and al., 2009)* (**Figure 15**).
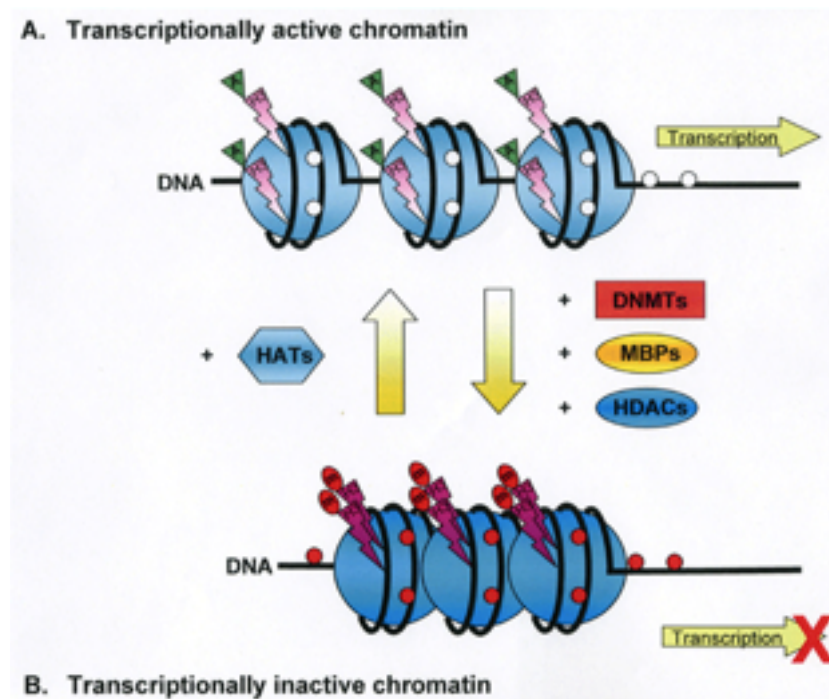
Figure 15: Epigenetic control of gene transcription by alterations in DNA methylation status and chromatin modifications.

A: Transcriptionally active DNA (light blue circles) is associated with unmethylated CpG residues on the DNA (white circles) and specific posttranslational histone modifications, including increased levels of acetylation (green triangles) of key histone H3 lysine residues (light pink arrows). The resultant DNA is readily accessible to histone acetyl transferases (HATs) as well as other enzymes and cofactors important in gene transcription. **B:** Chromatin may be transformed into a transcriptionally repressive conformation, which is characterized by methylated CpGs on the DNA

(red circles), compacted nucleosomes (dark blue circles), with deacetylated histones (dark pink arrows), and further posttranslational modifications, including methylation of specific histone H3 lysine residues (red ovals). This repressive conformation renders the DNA inaccessible to the transcriptional machinery, and is established and maintained by proteins, including methyl-CpG binding proteins (MBPs), histone deacetylases (HDACs), and DNA methyltransferases (DNMTs). *Lindsey and al., 2005*

During my PhD I got interested in the histone deacetylase and miRNA in gastric cancer. I will now speak in more detail of these two processes, first of all from a general point of view, and then in the context of GC.

2. The histone deacetylases

During our investigations on the compared mode of action of cisplatin and RDC11, we have highlighted the involvement of histone deacetylases (HDAC, Article 1). As described above, HDACs play an important role in the establishment of covalent histone modifications. They have the ability to remove the acetyl group of lysine previously transferred by the HAT on a vast array of nuclear and cytoplasmic protein.

2.1. Histone acetylation: mode of action

The histone acetyltransferase (HAT) catalyzes the transfer of an acetyl group from acetyl coenzyme A to the ε-amino groups of certain lysine residues in the tail of the histone proteins. Neutralization of the positive charge on the $NH3^+$ group of lysine will enable the inhibition of contact with DNA, adjacent nucleosomes and proteins. Consequently chromatin will have a more flexible conformation, making it more accessible to the transcriptional machinery. This process is reversible via the action of HDAC allowing the withdrawal of the acetyl group *(de Ruijter and al., 2003)* (**Figure 16**). The level of histone acetylation thus depends on the balance between the activity of HAT and HDAC. It plays a critical role in chromatin remodeling and regulation of gene transcription. Histone acetylation is preferably observed on histone H3 and H4 *(Ropero and al., 2007)*.
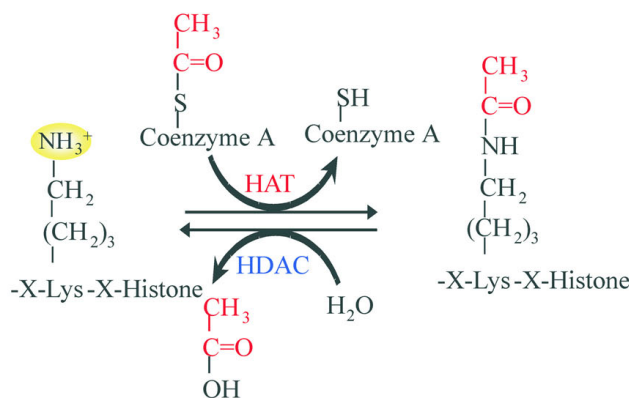
Figure 16: Role of HAT and HDAC in transcriptional regulation. *Kim and al., 2003*

2.2. HDAC classification

HDACs are part of the superfamily of the zinc metalloenzymes and are classified according to their sequence homology with the HDAC yeast *S. cerevisiae*. There are 18 HDAC in humans that are grouped into 4 classes, the classical HDAC of class I, II and IV whose activity is dependent on the Zn^{2+} ion (**figure 17**). The class III, called Sirtuin differs from the classical HDAC by their NAD^+

dependent mode of action. The latter will not be discussed in the descriptions that follow *(Parra, 2015; de Ruijter and al., 2003)*.
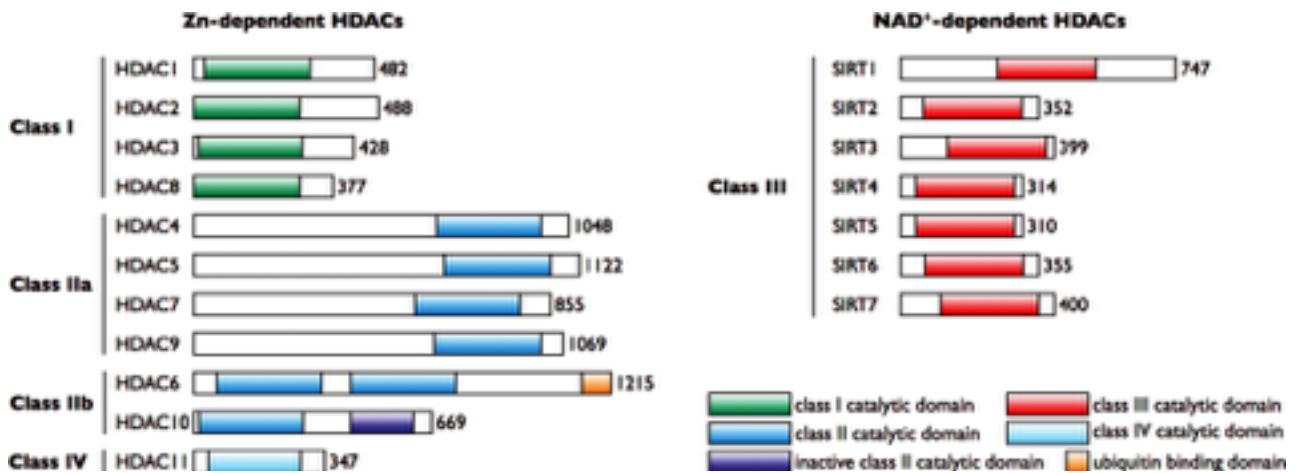
Figure 17 : Classification of HDAC enzymes. *d'Ydewall and al., 2012.*

Class I

The class I is represented by HDAC1, 2, 3 and 8 and relates to the yeast enzyme RPB3. Their catalytic domain present at the N-terminal occupies more than 50% of the protein. They are mainly localized into the nucleus. HDAC1 and HDAC2 have 82% of sequence homology *(Wade, 2001; Cress and al. 2000; Li and al., 2002; Kao and al., 2000)*. Their activity is dependent on the formation of co-repressor complex after their phosphorylation, allowing their interaction with the components of the complex mSin3A and/or NuRD *(Galasinski and al., 2002; Zhang and al., 1999; Ashburner and al., 2001)*. Conversely, HDAC3 does not interact with these complexes. However, they all also have the ability to directly interact with DNA binding proteins such as YY1, SP1 and RbBP. HDAC3 has a non-conserved C-terminal end which is important for its deacetylase and transcriptional repression activity. It has an import (NLS) and export (NES) nuclear domain. The balance between the two is dependent on the cellular context *(Yang and al., 2002a)*. HDAC3 has the ability to form complexes with SMRT / NCoR that are necessary for its activity through their deacetylation domain *(Berthos and al., 2001; Kao and al., 2002)*. Furthermore, HDAC3 plays an important role in the complex formation with other HDAC, including HDAC4 and HDAC5 from the class II *(Fischle and al., 2001)*. Finally, HDAC8 has sequence similarity with HDAC3. Little is known about this member.

Class II

The class II is divided into 2 sub-family, consisting of the Class IIa (HDAC4, 5, 7 and 9) and the Class IIb (HDAC6 and 10).

Class IIa

They are tissue specific, and described in cardiac and smooth muscle, bone, immune system, vascular system and the brain *(Parra, 2015)*. In the 2000s, Olson and his colleagues have made knockout of each member of the family which helped to highlight their crucial role in the phenomenon of development and differentiation *(Vega and al., 2004)*. They may have a nuclear or cytoplasmic localization. They have a catalytic domain in their C-terminal end. Their N-terminal end consists in a NLS domain, and in binding domains for CtBP, MEF2, 14-3-3, unique characteristic differentiating them from other HDACs *(Berthos and al., 2001)*. Moreover HDAC5 also has a NES domain. They all have the ability to interact with co-repressor complex like SMRT / NCoR. They were particularly described for their interaction with the MEF2 factor, which plays a crucial role as a DNA-binding protein in muscle differentiation *(McKinsey and al., 2001)*. The analysis of this process allows a better understanding of their shuttling between the nucleus and cytoplasm (**Figure 18**).
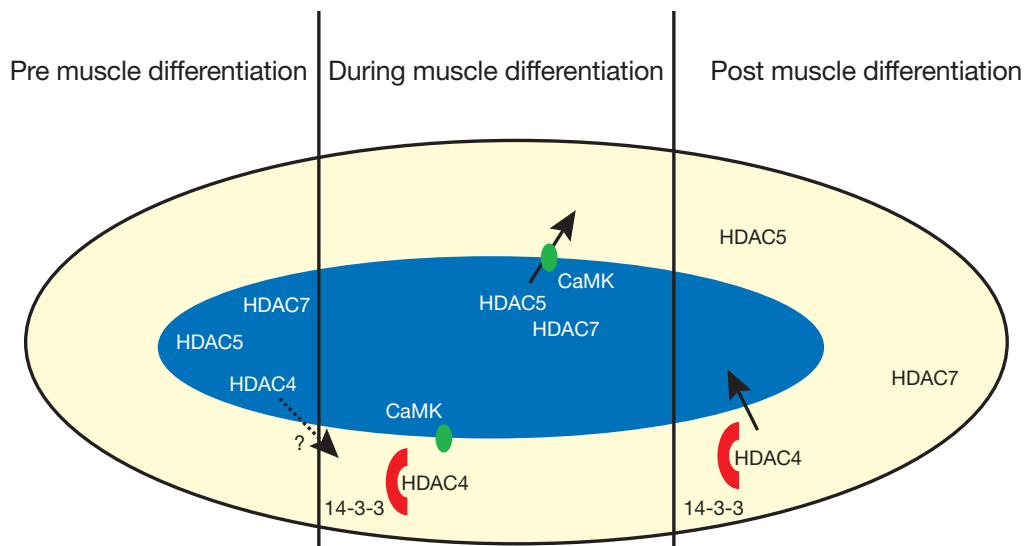
Figure 18: Shuttling of HDAC4/5/7 during muscle differentiation. *de Ruijter and al., 2003*

The interaction between HDAC4/5/7 and MEF2 in the nucleus will induce its repression and therefore a block in muscle differentiation. The CAMK will help to inhibit and disrupt the complex HDAC / MEF2 due to the HDAC phosphorylation. The export of HDACs is done via the cellular

export factor CMR1. HDAC4 is maintained in the cytoplasm by interaction with the 14-3-3 factor *(Guenther and al., 2001, Andreucci and al., 2002; McKinsey and al., 2000)*. Interestingly, HDAC4/5/7 can make the connection between HDAC3-SMRT / NCoR. Thus they show a capacity to fine-tune the gene repression function change of location dependent stimuli *(Fischle and al., 2002)*. HDAC9 has its catalytic domain at the N-terminal. Various splicing variants are described (HDAC9a / b / c), but others are suspected. Like the other members of the class IIa, it has the ability to interact with MEF2, indicating an important role in the differentiation of muscle *(Zhou and al., 2001; Zhou and al., 2000)*.

Class IIb

HDAC6 has two catalytic domains in tandem and a ubiquitin binding domain. It is mostly found in the cytoplasm. It can target the tubulin and therefore has an important role in the regulation of tubule-dependent cell motility *(Hubbert and al., 2002)*. HDAC10 has a complete catalytic domain in N-terminal and a second incomplete and inactive in C-terminal. It has two Rb-binding protein domain, which can confer on it a role in cell cycle regulation. It was described to interact with many HDACs except HDAC6 *(Fischer and al., 2002; Guardiola and al., 2002; Tong and al., 2002)*.

Class IV

HDAC11 has phylogenetic homology with the HDAC from class I and II and has a catalytic domain in N-terminal. It has not been described for its interaction with co-repressor complex *(Gao and al., 2002)*.

2.3. Role of HDAC

Acetylation was the first post-translational modification of histones to be identified and characterized. 50 years ago, Allfrey and colleagues made the discovery of the role of HDAC in the regulation of gene expression *(Allfrey and al., 1964)*. The regulatory network of the HDAC involves *(Ropero and al., 2007; Li and al., 2014)*:

- They can repress the transcription through the stabilization of the DNA/histones interaction
- Their ability to form co-repressor complex allowing them to recruit histone modifiers and therefore to regulate the chromatin based process.
- They can regulate non-histone protein, like transcription factors (p53, E2F).

Role of co-repressor complex

HDACs are part of co-repressor complex (mSin3, NCoR and SMRT). The formation of these complexes will allow HDACs to be directed at specific genomic regions by interacting with DNA binding factor such as transcription factors, nuclear receptors and epigenetic modifiers (MBD DNMT and HMT) (*Glass and al., 2000*). HDACs thus allow through their deacetylase activity to stabilize the silencing of certain genes introduced by DNA methylation for example. The DNMT have been described for their ability to recruit HDACs at the methylated DNA. Indeed, deletion of DNMT1 in cancer cells leads to an increase in H3 acetylation and reduced methylation. Furthermore a loss in interaction between HDAC and the heterochromatin protein (HP1) with H3 is observed *(Espada and al., 2004)*. HDAC2 is involved in neuronal differentiation by direct interaction with DNMT3b; treatment of PC12 cells with a HDAC inhibitor allows a decrease in the differentiation while overexpression of DNMT3b promotes differentiation *(Bai and al., 2005)*.

Non-histone protein substrates

HDACs have the ability to target more than fifty non-histone proteins. The first acetylated non-histone protein to have been characterized is p53 *(Yao and al., 2011)*. Since then, many transcription factors, as well as cytosolic proteins have been described as referenced in the table below (Table 1).

Classe	HDACs	Subcellular localisation	Non-histone proteins
I	HDAC1 HDAC2 HDAC3 HDAC8	Nucleus	p53, STAT1, YY1, ERa, SP1, MyoD, E2F-1 STAT3, YY1 SRY, STAT3, YY1, SHP, GATA1, GATA2, RelA —
IIa	HDAC4 HDAC5 HDAC7 HDAC9	Nucleus / cytoplasm	GATA1 GATA1 — —
IIb	HDAC6 HDAC10	Cytoplasm Nucleus / Cytoplasm	SHP, α-Tubuline, Hsp90 —
IV	HDAC11	Nucleus / cytoplasm	—

Table 1: Non-histone proteins, according to *Zhang and al., 2014b*.

Depending on their non-histone target HDAC will have a role in the DNA binding of the protein, the protein / protein interaction, the protein stability and the protein translocation *(Kim and al., 2003)*. For example, the interaction of the tumor suppressor Retinoblastoma (Rb) with HDAC1 / 2 will allow the repression of promoters containing E2F, Rb being itself directly recruited by the E2F

protein and thus modulate the G1 / S transition of the cell cycle *(Luo and al., 1998)*. HDACs have also the ability to interact with nuclear receptors such as estrogen receptor (ER). HDAC1 interacts strongly with ER, which leads to the repression of its activity in absence of estrogen through the inhibition of its target gene transcription *(Kawai and al., 2003)*. Furthermore, acetylation of p53 can stabilize the protein by preventing its ubiquitination on the same lysine residues. The complex HDAC1/NuRD causes the deacetylation of p53 and therefore its degradation by the proteasome *(Juan and al., 2000; Luo and al., 2000)*. A genome-wide analysis of acetylated proteins highlighted their role in many biological processes such as proliferation, apoptosis, differentiation and angiogenesis *(Choudhary and al., 2009)*.

2.4. Therapeutical interest of HDAC inhibitors treatment in cancer

The interest in HDAC has been growing the last few years for two reasons *(Minucci and al., 2006)*:

a. Their expression has been linked to carcinogenesis. Indeed, an alteration in HDAC expression is observed in numerous hematological malignancies and solid tumors.

b. They are of interest in cancer therapy in view of their reversible activity with the use of an HDAC inhibitor (HDACi).

Fraga et al. have reported a loss in acetylation of the histone H4K16 and trimethylation H4K20 at repeated DNA sequences of tumor cells in early stage in tumorigenesis *(Fraga and al., 2005)*. This early epigenetic modification represents a cancer signature. Hypoacetylation in colon, breast, ovarian, stomach cancer is observed. The level of histone hypoacetylation is correlated with tumor initiation. Two hypotheses are possible to explain this process, the alteration of the expression of HAT by inactivating mutations or chromosomal translocation and an aberrant expression of HDACs, which is developed in the following section *(Ropero and al., 2007)*.

HDAC and cancer

The architectural model for the involvement of HDACs in cancer is the acute promyelotic leukemia (APL). 100% of patients with APL exhibit fusion proteins with the retinoic acid receptor (RAR), as RAR-PML (promyeloticytic leukemia) and RAR-PLZF (PML-zinc finger). Under normal conditions, in the absence of retinoic acid (RA), the RAR is present on its response elements with HDAC to repress transcription its target genes. In the presence of RA, there is a disruption of the complex RAR / HDAC and a transcriptional activation of its target genes *(Lin and al., 2001)*. Fusion proteins allow the stabilization of the RAR / HDAC complex regardless of the presence in RA, as well as the recruitment of other factors involved in chromatin remodeling such as DNMT and HMT. One of the major reported effects is the degradation of p53, because of its hypoacetylation and therefore its

instability, promoting tumor development. HDAC therefore plays a critical role in establishing the tumor phenotype. Thereafter, the altered expression of certain HDACs was put forward in many solid tumors. For example, overexpression of HDAC1 is observed in tumors such as gastric, breast, prostate cancer and correlated with poor prognosis *(Choi and al., 2001; Zhang and al., 2005; Halkidou and al., 2004)*. However, the expression of HDAC1/3 is correlated with that of the estrogen and progesterone receptor in breast cancer, which contribute to their interest as an independent marker of prognosis *(Krusche and al., 2005)*. The expression of HDAC8 is increased in a cancer specific manner, as in the childhood neuroblastoma and correlates with a poor prognosis and survival for the patient *(Oehme and al., 2009)*. In view of these factors, the contribution of HDACs in carcinogenesis has been studied (**Figure 19**).
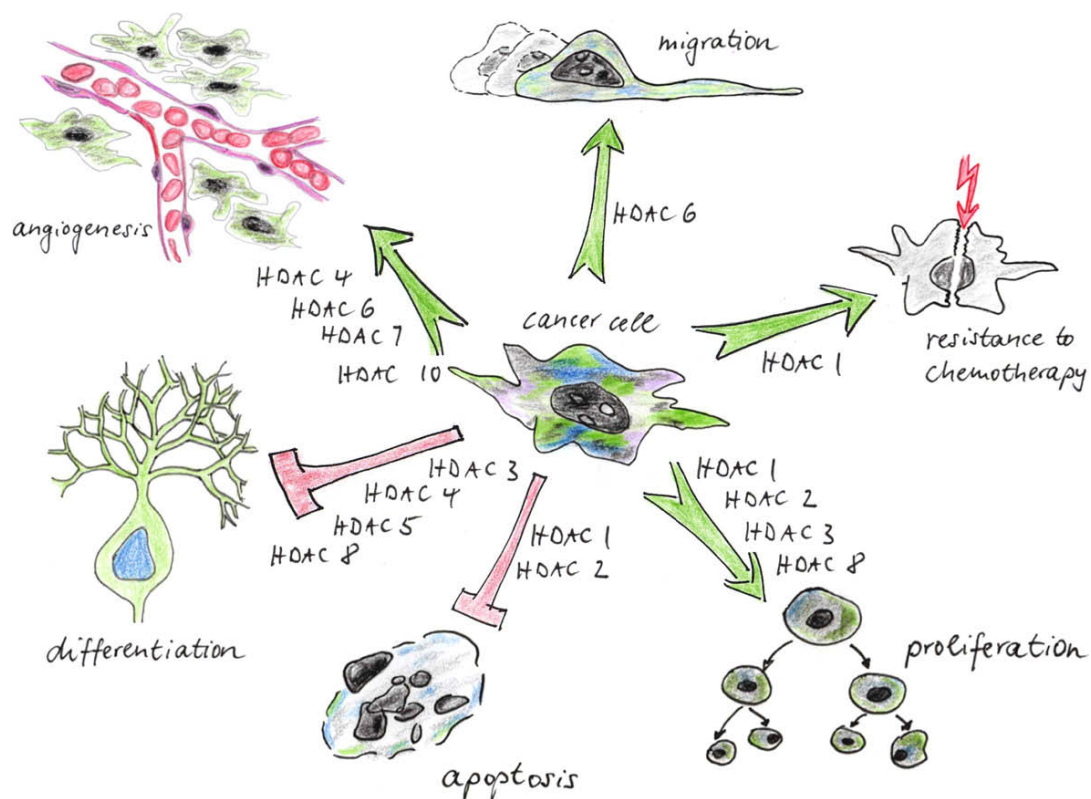
Figure 19: HDAC family members control hallmarks of cancer cell biology. Selective targeting of individual HDACs cause differentiation, apoptosis, cell cycle inhibition, inhibition of migration, susceptibility to chemotherapy and anti-angiogenesis. *Witt and al., 2009*

HDAC inhibitors

Involvement of HDAC in carcinogenesis together with the reversibility of their actions makes them good therapeutic candidates. HDAC inhibitors (HDACi) have shown cytotoxic effects in a broad range of tumor cells. Many natural or synthetic HDAC inhibitors have been developed. They are

classified according to their structure and allow the inhibition of HDAC class I and II by competition with the Zn^{2+} ion critical for the enzyme activity. A dozen of them are currently in clinical trials, but only the SAHA (Vorinostat) is FDA approved for the treatment of cutaneous T-cell lymphoma (CTCL). Furthermore many studies have been carried on the combination of HDACi with cytotoxic agents such as platinum salt, taxane, topoisomerase inhibitors, and also with methyltransferases inhibitors, showing synergistic effects of the combination of different drugs *(Marchion and al., 2007; Chueh and al., 2014; Liu and al., 2006)*. Among the most cited HDACi, there are:

- Short-chain fatty acid : Valproic acid (VPA), sodium butyrate (NaB)
- Cyclic tetrapeptide : Depsipeptide
- hydroxamic acid : SAHA, Trichostatin A (TSA), LAQ824
- Benzamide : MS275

Generally, the HDACi lead to an overall increase in histone and transcription factors acetylation, but they also allow the decondensation of the chromatin. They have the ability to modulate various biological processes such as cell cycle arrest, inhibition of growth, differentiation and apoptosis implicated in the carcinogenesis. In the following section I became interested in the impact of HDAC in these different pathways involved in cancer, allowing an overview of the therapeutic value of HDACi.

Apoptosis

HDAC1/2 have been described for their role in the regulation of apoptosis. Indeed, inhibition of HDAC1/2 by SAHA and TSA treatment of colon cancer cells allows the induction of p53-dependent apoptosis through hyperacetylation and therefore stabilization of p53. The HDACi allows the induction of some pro-apoptotic gene (*BAX*, *PUMA*) and the reduction of anti-apoptotic genes (*BCL2*) *(Medina and al., 1997; Sawa and al., 2001)*.

DNA damage repair

HDACs have an important role in chromatin remodeling, especially in the repair mechanisms of DNA damage via the direct regulation of repair proteins. They are involved in the current chemotherapy resistance mechanisms by promoting repair of DNA damage. HDAC1/2 are actively recruited at the DNA lesions to promote their repair via ATM. The deletion of HDAC1/2 allows to sensitize tumor cells to chemotherapy. Similarly deletion of HDAC3 and HDAC4 reduces DNA damage repair *(Miller and al., 2010; Bhaskar and al., 2010)*. Indeed, HDAC4 is involved in cisplatin resistance in ovarian cancer cells by allowing the activation of STAT1 and thus the activation of DNA repair *(Stronach and al. 2011).*

Cell cycle control

HDACs are key regulators in the DNA replication. They can regulate the cyclin-dependent kinase (CDK), important checkpoint of the cell cycle. In addition as previously described HDAC1 / 2 have the ability to modulate the transition from the G1 / S phase by the interaction with the Rb factor, while TSA treatment abrogates this repression *(Brehm and al., 1998; Luo and al., 1998)*. Moreover HDAC4 has a dual role in regulating p21, a key factor in cell cycle arrest mediated by p53. Indeed in colonic crypts HDAC4 interacts with SP1 for the transcriptional repression of p21 and Bax (anti-apoptotic factor) promoting survival and cell proliferation. In contrast, in response to the stress of DNA damage, p53 has the ability to recruit HDAC4 to induce the expression of p21 and thus DNA repair *(Mottet and al., 2009; Wilson and al., 2008)*.

Autophagy

Autophagy is a process of self-degradation of unnecessary or dysfunctional cellular components via the lysosome machinery that can be regulated by HDAC. HDAC6 and HDAC10 have been described to promote autophagy and thus allow cell survival in response to chemotherapy *(Lee and al., 2010; Oehme and al., 2013)*.

Metabolism and senescence

HDAC1 has the capacity to regulate AMPK, a metabolic sensor and enable its activation *(Lin and al., 2012)*. Deletion of HDAC3 allowed to highlight its involvement in the regulation of genes involved in fatty acid metabolism, in the use of glucose, oxidation and phosphorylation through the activation of PPARγ and the mTOR pathway *(Fajas and al., 2002; Knutson and al., 2008)*.

Chaperone function

HSP90 is described to favor structural maturation of proteins and to protect them against proteosomal degradation. Its deacetylation by HDAC6 enables its function in tumor cells through the protection of the VEGF receptors, VEGFR1/2 *(Kovacs and al., 2005; Bali and al., 2005)*. Moreover, HSP90 is known to have the ability to interact with the p53 mutant and some fusion proteins (leukemia characteristic) and thus prevent their degradation *(Yang and al., 2006)*. This interaction is decreased with the depsipeptide treatment *(Yu and al., 2002)*.

Angiogenesis

HDACs play an important role in angiogenesis through regulation of hypoxia factors HIF1A and VEGF. Their recruitment is dependent on cellular contexts like hypoxia, hypoglycemia and serum starvation. HDAC4/6 have the ability to interact with HIF1A and prevent its proteosomal

degradation by inhibiting its interaction with VHL protein, an E3-ubiquitinase *(Qian and al., 2006)*. The formation of HDAC7 / HIF1A complex allows its translocation to the nucleus and activation of its target genes *(Kato and al., 2004)*. HDACi treatment allows the decrease of neovascularisation (Depsipeptide, SAHA) *(Kwan and al., 2001).*

Limits and outlooks for HDACi

Only a small percentage of gene (between 0.5 and 20%) is affected at the transcriptional level in response to the HDACi. Furthermore, only a limited number of developed HDACi have shown a real interest in early clinical trials. This lack of significant response may be explained by the inclusion of patients with advanced refractory tumors pretreated with intensive chemotherapies.

Marchion have highlighted three issues that remain unresolved to help improve therapies with HDACi *(Marchion and al., 2007)*:

a. Which HDAC is important in the biological effects observed in response to HDACi?

b. Does more than one HDAC contributes to an individual effect as what has been described with HDAC1 / 2 and the capacity of HDAC3 to form co-repressor complex?

c. Does HDACs have redundancy in their function?

The HDACi are pan-inhibitors against the class I and II, but each has a greater affinity for one or the other HDACs. For example, the TSA preferentially inhibits HDAC1/3/8, whereas the VPA mainly target HDAC1/2. Thus the level of expression of HDAC will dictate the sensitivity of tumors to treatment. This idea is supported by the study of Ropero with the HDAC2 mutation in colon cancer cells, which exhibit a response to TSA but not to VPA *(Ropero and al., 2007)*. Thus, lack of cellular markers to predict the effect of the drug leads to an arbitrary selection of patients and therefore non-objective for clinical benefits.

3. The microRNA pathway

MicroRNAs (miRNAs) are small noncoding single-stranded RNA of 22 nucleotides in length. They have the ability to target the 3 'UTR region of some messenger RNAs (mRNA) to inhibit their translation. This mechanism is highly conserved across species *(Selbach and al., 2008; Lim and al., 2005)*. The first miRNA being described in *C. elegans* is Lin-4 by Lee and colleagues in 1991 for his involvement in the developmental process *(Lee and al., 1991)*. Currently, more than 2,500 humans miRNAs have been described according to the miRBase database *(Kozomara and Griffiths-Jones, 2014)*. Each miRNA induces post-transcriptional repression of dozens or even hundreds of mRNA. This is explained by the presence of a highly conserved recognition sequence of mRNA in 5' ends of miRNAs (the seed sequence, 2 to 7 nucleotides). Thus, about 60% of protein-coding genes have the capacity to be regulated by miRNAs *(Bartel and al., 2009)*. Olena describes the genomic organization of genes encoding microRNAs *(Olena and al., 2010)*:

- Within a protein-coding gene, the sequence encoding for one or more miRNA is located in an intron and processed after splicing. In this case, the transcription is dependent on the host gene promoter. However, several transcription starting site are described for miRNAs highlighting the possibility of a distinct promoter for intronic miRNAs.

- Exclusive gene encoding one or more miRNA (then forming a cluster) and under the control of its own promoter.

- More rarely, the sequence encoding a miRNA covers both intron and exon of a host gene, the maturation process remains unclear at this time *(Rodriguez and al., 2004)*.

The study of the functional organization of the genome revealed that more than a third of the genes encoding miRNA are clustered *(Griffiths-Jones and al., 2008)*. These clusters thus encode a very long transcript (up to 1 kb), which after its maturation produce several different miRNA sequences. The miR-100 / let-7 / miR-125 miRNA cluster was the first one described for their common action in animals bilateral development *(Ha and al., 2014)*. According to Yuan, miRNAs in the same cluster will target member of the same functional protein group *(Yan and al., 2009)*.

3.1. Biogenesis of miRNA

Lee et al. showed that transcription of miRNAs is mainly dependent of the RNA polymerase II (Pol II) *(Lee and al., 2004)*. Biogenesis of miRNAs involves two steps: a nuclear and cytoplasmic process of the precursor by two ribonuclease III endonucleases, Dicer and Drosha (**Figure 20**).
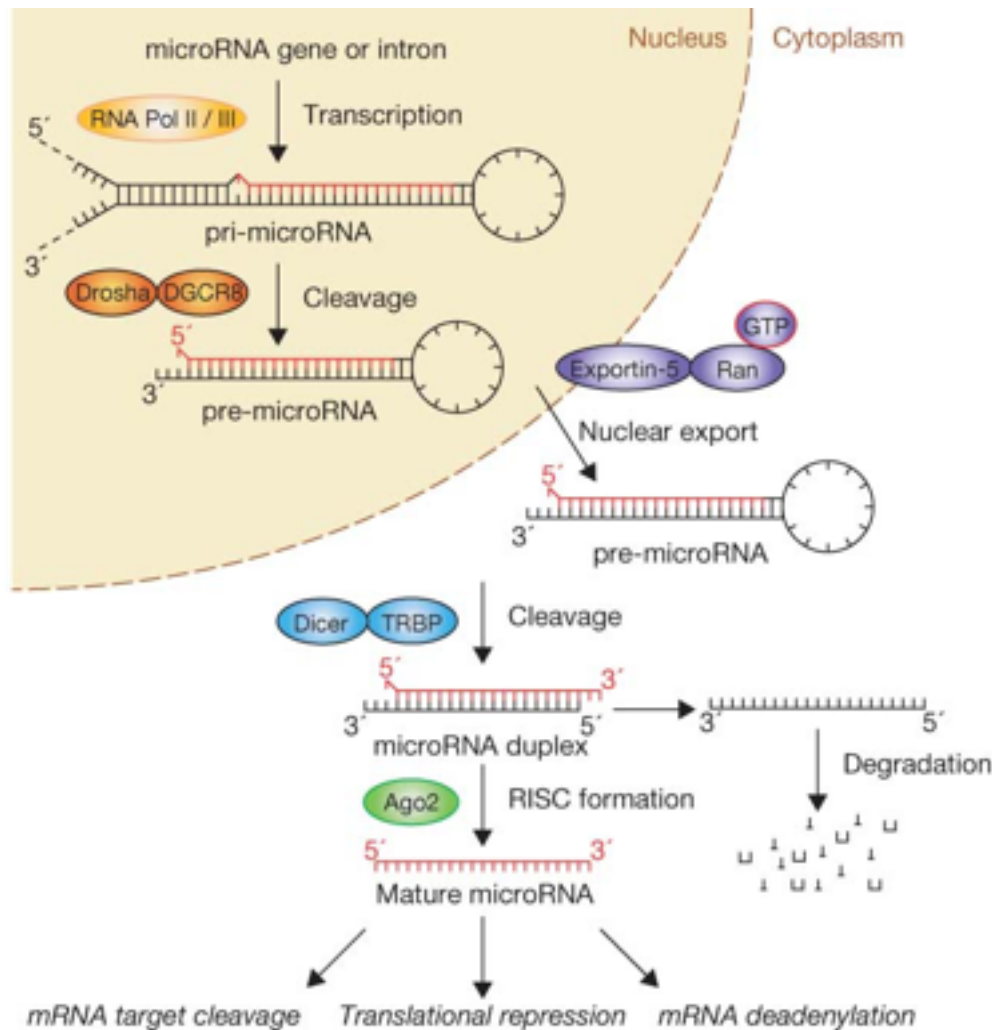
Figure 20: miRNA biogenesis. *Winter and al., 2009*

Nuclear step process

The transcription of genes coding for miRNAs leads to the formation of a first long precursor, **the pri-miRNA**, with a local stem loop containing the mature miRNA sequence, and two single strand RNA in the 5' and 3' end *(Bartel and al., 2004)*. The pri-miRNA will be cleaved by the microprocessor composed of the RNase III Drosha, and DGRC8 (DiGeorge syndrome critical Region 8) involved in the recognition of double-strand RNA (dsRNA). A cross regulation between the two partners is observed. DGRC8 has the ability to stabilize Drosha by protein-protein interaction. Conversely, Drosha is able to downregulate DGRC8. The germline Drosha and / or DGRC8 deficiency induces early embryonic lethality, demonstrating their importance in development. In human, genomic deletion of DGRC8 leads to a neurological disorder, the DiGeorge syndrome. DGRC8 will therefore allow the recognition of dsRNA positioning Drosha for its endonuclease activity *(MacFarlane and al., 2010)*. Cleavage of the pri-miRNA will allow the release of the stem loop structure and the formation of the **pre-miRNA** hairpin precursor of 60-70 nucleotides *(Han and al., 2006)*. The pre-miRNA will be translocated into the cytoplasm by an active mechanism

involving the Ran-GTP / Exportin 5 (EXP5) complex *(Lund and al., 2004)*. In addition to allowing its export EXP5 provides protection of the pre-miRNA against nucleolytic attacks. Modulating the export of precursor EXP5 is a key step in the regulation of miRNA biogenesis *(Bohnsack and al., 2004; Zeng and al., 2004)*.

Cytoplasmic step process

The pre-miRNA is recognized by Dicer, another RNase III. It will allow the cleavage of the precursor at its terminal loop and thus the generation of an **RNA duplex** of about twenty nucleotides *(Lee and al., 2003)*. The pre-miRNA processing complex is composed of Dicer, TRBP (TAR RNA-Binding protein) and PACT. TRBP modulates the process efficiency of the pre-miRNA by Dicer and increase its stability. TRBP and PACT will serve as mediators for the association of the Dicer-RNA duplex complex with the Argonaute (Ago) protein family *(Chendrimada and al., 2005; Lee and al., 2006)*. Moreover these two proteins can prevent the activation of PKR (Interferon induced ds-RNA activated protein kinase receptor) by the presence of the cytoplasmic pre-miRNA *(Perron and al., 2008; Lee and al., 2006; Rossi and al., 2005a)*. After Dicer cleavage, the RNA duplex is dissociated, keeping only the mature strand called the "guide" strand who will associate with one of the four Ago proteins *(Salzman and al., 2007; Robb and al., 2007)*. Dissociation of the RNA duplex could be done by Dicer and/or Ago, but others proposed the hypothesis of a combination of helicase proteins (yet to be characterized) to the processing complex. A model has been proposed based on work performed in Drosophila, where the choice is not random but is dependent on the less thermodynamically stable 5' extremity of the two strands *(Khvorova and al., 2003)*. However, some studies also showed that the strand selection can depend on the cellular or tissue context *(Schwarz and al., 2003)*.

There are 4 Ago proteins described in mammals (Ago1-4) *(Höck and al., 2008)*. Pillai et al. have developed a model in which the Ago2 protein is fused with a specific recognition sequence (B-box binding site) targeting the mRNA of luciferase at its 3'-UTR region, where a variable number of B-box binding site was inserted *(Pillai and al., 2004)*. The direct interaction of the Ago2 protein to the 3'-UTR of the mRNA by the B-Box binding site leads to the repression of the luciferase expression. Furthermore, this repression is proportional to the number of recognition sites inserted at the mRNA level. This study therefore showed that this inhibiting function requires the binding of the Ago2 protein to the mRNA target while the miRNA appears to have only a guiding function to the target mRNA.

Once bound to its target RNA, the miRNA - Ago2 complex recruits a third protein: GW182 (glycine-tryptophan repeat-containing protein of 182 kDa), forming the RNA induced silencing complex (RISC). Indeed, deletion of GW182 and inhibition of the formation of the Ago2 - GW182 complex both lead to a lack of mRNA translation suppression by the miRNAs *(Fabian and al., 2012)*. GW182 is a major component of the P-body, an essential structure for the functionality of the miRNAs. The miRNA will therefore guide the RISC complex at the 3'UTR end of its target mRNA, where it is fixed through its seed region. In some cases, a perfect match between the miRNA and its target RNA allows Ago2 to cleave the mRNA by its endonuclease (slicer) activity. In the case of a non-perfect , seed-mediated, match, the mRNA translation is inhibited during the initiation or elongation phase of the translation *(Bartel and al., 2004; Yekta and al., 2004)*. Inhibition of the translation may also be the result of a spatial separation of the mRNA with the translational machinery, resulting in sequestration of the mRNA in the P-body. In addition to translation initiation inhibition, there is another mechanism resulting in the down-regulation of the targeted mRNAs. Indeed, RISC can recruit the deadenylation complex formed by the protein CCR4 and NOT. mRNA deadenylation can lead firstly to the linearization of the mRNA, which therefore loses its ability to be translated in a cap-dependent manner. Secondly, RISC recruits decapping enzymes DCP2 and DCP1 that lead to the degradation of the mRNA by the exoribonuclease Xrn1 *(Huntzinger and al., 2011)*. It is now known that the two mechanisms, inhibition of translation initiation, and mRNA decay, are coupled with the former happening first, followed by the later *(Bazzini and al., 2012, Eichhorn and al., 2014)*.

3.2. Implication of miRNA in cancerogenesis

The regulation of miRNAs has been heavily studied in recent years for their involvement in carcinogenesis. Indeed in view of the number of their target mRNAs, miRNAs are involved in the majority of biological processes including differentiation, proliferation and apoptosis. However, it is difficult to determine whether miRNAs can cause and promote the development of cancer or if they are just a consequence of the perturbations associated with it *(Krek and al., 2005)*. An alteration of the transcriptional machinery of miRNAs is generally observed in cancers, resulting in an overall reduction of their expression correlated with poor prognosis *(Hayes and al., 2014)*.

Biogenesis Alteration

Biogenesis of miRNAs is a mechanism that is finely regulated, and its alteration leads to the development of disease such as cancer and neurological disorder (DGRC8 and DiGeorge Syndrome). The processing of Drosha is controlled by many RNA-binding protein such as p68 or

SMAD (1-4) and R-SMAD. The bone marrow protein (BMP) has the ability to favor the interaction of R-SMAD and p68 and thus increase the Drosha processing efficiency for miR-21, an oncogene *(Ha and al., 2014)*. The EXP5 mutation in some tumors leads to an overall decrease in the expression of miRNA *(Melo and al., 2010)*. On the other hand, mutations in TRBP have been reported in many cancer causing destabilization of Dicer and therefore a reduction in the maturation of miRNAs *(Melo and al., 2009).*

miRNA: OncomiR and Tumor suppressor

Many miRNAs have been described to be either tumor suppressor or oncogenic (oncomiR). Like other genes involved in tumor initiation and progression, miRNAs can be deregulated by the amplification and deletion of their sequence / promoter but also by epigenetic changes and transcription factors *(Hayes and al., 2014)*. Thus, there is a decrease in the expression of tumor suppressor, and an increase of the expression of oncomiR. The miR-15/miR-16 cluster was the first to be described for its role in cancer in 2002 *(Calin and al., 2002)*. In fact it is frequently deleted in chronic lymphocytic leukemia (CLL), which results in an increased expression of their target gene Bcl2, an anti-apoptotic factor. They also have a role in the chemosensitivity of CLL tumors. Members of the Let-7 family are also frequently altered in cancer such as lung, colon and pancreas cancer *(Johnson and al., 2005)*. They have the ability to target the oncogenes Ras and HMGA2 that have a role in the transformation and proliferation of tumor cells *(Bos, 1989)*. Conversely, miR-21, a common oncogenic miRNA, is overexpressed in numerous cancers such as breast, colon, stomach cancer. It targets, among others genes, the tumor suppressor PTEN thereby affecting tumor growth, but also the invasion and metastatic development. Loss of PTEN expression leads to the activation of the Akt pathway and thus cell survival, but also allows an increase of the FAK factor involved in cellular motility. In addition the expression of miR-21 is correlated with resistance to treatment of 5-FU in colon cancer *(Meng and al., 2007; Zhu and al., 2008; Park and al., 2002)*. Finally, an increase in miR-21 21 and decrease in Let-7 expression have been associated with clinical outcome in colorectal and breast cancer *(Pan and al., 2010)*.

Furthermore, some transcription factors have the capacity to induce the expression of oncomiR. In particular, c-myc is known to induce the expression of the miR-17/miR-92 cluster, which in turn modulates E2F1 and results in increased tumor development *(He and al., 2005; O'Donnell and al., 2005)*. Similarly, NF-kB which induces the expression of miR-146a to inhibit the signaling pathway of TLRs and thus the immune response *(Taganov and al., 2006)*.

Epigenetic modifications and miRNA in cancer

As I have already mentioned, epigenetic modifications such as DNA methylation and histone modifications play a major role in cancer development. Recently, miRNAs have also been characterized as key actors in the epigenome regulation *(Chuang and al., 2007).* Indeed, their important roles in the developmental process (for example Let-7) and determining cell identity make them key players in the development of epigenetic phenotypes *(Saetrom and al., 2007)*. Different teams have shown that miRNAs were involved in the regulation of both DNA methylation and histone acetylation levels in the establishment of cancer. Maison et al. showed that an RNase treatment could inhibit the colocalisation of the H3K9 methylation with HP1 at the pericentromeric chromatin *(Maison and al., 2002)*. Moreover, Fukagawa et al. described a potential implication of Dicer in the formation of the heterochromatin, although this was done in an artificial setting *(Fukagawa and al., 2004)*. Ago1 and Ago2 may also be involved in the dimethylation of H3K9 and so the establishment of gene silencing *(Kim and al., 2006; Janowski and al., 2006)*. Furthermore, many examples demonstrate that miRNA expression can be controlled by DNA methylation and histone modifications, such as their deacetylation, but especially miRNAs have the ability to modulate both mechanisms *(Rusek and al., 2015)*. miR-127 is the first miRNA to have been described for its epigenetic regulation in cancer by Saito *(Saito and al., 2006)*. Since this first reported example, over 45% of miRNAs have been shown to be regulated at the epigenetic level. In many cancers, such as breast cancer, expression of miR-127 is greatly reduced in comparison with the healthy tissue. It is involved in the development of apoptosis by allowing the inhibition of BCL6, an anti-apoptotic factor. Treatment of cancer cells by 5-Aza-CdR (a methylation inhibitor) combined with an HDACi allows to recover its expression *(Egger and al., 2004)*. This experience highlights the involvement of the CpG island hypermethylation on miRNA promoters with a tumor suppressor activity and so their transcriptional repression. Interestingly, in the case of intronic miRNAs, CpG island upstream of their sequence are frequently found, hypothetically corresponding to their own promoter, and thus subjected to be controlled by methylation. Interestingly, in the case of intronic miRNAs CpG island are frequently found upstream of their sequence, hypothetically corresponding to their own promoter *(Lyle and al., 2000; Wutz and al., 1997)*. Conversely, some miRNA with oncogenic activity exhibit demethylation of their promoter. This is the case for miR-196a, which surexpression in lung cancer correlates with advanced clinical state and lymph node metastasis development *(Rusek and al., 2015).*

Furthermore, histone modifications including the HDACs also play a role in suppressing the expression of miRNAs *(Swierczynski and al., 2015)*. For example, HDAC1 has the ability to inhibit transcription of miR-34 and miR-449a in prostate cancer *(Noonan and al., 2009; Nalls and al.,*

2011). miR-34 is involved in a positive feedback loop with p53 in the control of p21 expression, important in cell cycle regulation. Further evidence of the regulation of miRNA by HDAC is repression of miR-183 by HDAC2 in neuroblastoma. This miRNA targets MYCN, which is important in the establishment of an invasive phenotype and the development of metastasis. Treatment with HDACi allows the overexpression of miR-183 following the hyperacetylation of its promoter *(Lodrini and al., 2013)*. Conversely miRNAs also have the ability to regulate these two mechanisms, called the epi-miRNA. The DNMT enzymes, responsible for DNA methylation, are predicted miRNA targets. Indeed, the miR-29 family is capable of inhibiting the translation of DNMT *(Rajewsky and al., 2006)*. In lung cancer, miRNAs from this family are often under-expressed, which allows the repression of many tumor suppressors. The miR-101 and miR-138 target EZH2, a member of the polycomb repressive complex, involved in H3K27 methylation. Decrease of the miRNA expression in NSCLC (Non-Small Cell Lung Cancer) causes the overexpression of EZH2 and therefore an increase in cell proliferation, decreased apoptosis and higher metastatic development *(Cho and al., 2011; Zhang and al., 2013a)*. Finally, a number of miRNAs have the ability to target different HDACs as described in the review of Swiercynski *(Swierczinski and al., 2015)*. His team referenced all articles describing a direct or indirect interaction between HDACs and miRNAs, identifying the target genes and the signaling pathways involved in the cancer. Part of their research is summarized in the table below (Table 2).

The involvement of miRNA in epigenetic changes especially with the emergence of those characterized for their oncogenic or tumor suppressor activity make them good therapeutic candidates for the treatment of cancers.

miRNA	HDAC	Target	Pathways	Pathology
miR-34	HDAC1	p21	Cell cycle arrest	Prostate cancer
miR-155	HDAC4	Blc6	Cell survival / Proliferation	B-cell lymphoma
miR-2861	HDAC5	BMP2	Osteogenesis	Osteoporosis
miR-433	HDAC6	a-Tubulin	Skeletal development	X-linked dominant chondrodysplasia
miR-140 5p	HDAC7	ADAM10	Migration	TSCC
miR-93	HDAC8	CCDN2	Proliferation	Colorectal cancer

Table 2: Liste of HDACs targeted by miRNA adapted from *Swiercynski and al, 2015*

Clinical and Therapeutic interest

Lu et al. showed that the miRNA expression profile could help to classify human cancers, better than other existing approaches. Indeed this expression profile known as miRNA signature could be useful in the diagnosis, prognosis, treatment response, to predict treatment efficiency and metastatic development *(Lu and al., 2005)*. This has already been established with lung cancer, which presents a unique miRNA expression profile allowing to define the prognosis *(Yanaihara and al., 2006)*. Another example is the over-expression of miR-21 in colon adenocarcinoma inducing a resistance to 5-FU treatment *(Schetter and al., 2008)*. Moreover, miRNAs have a significant interest in these areas in view of the existence of circulating miRNA in body fluids (plasma, urine, saliva). The presence of miRNA in these fluids is due to their secretion in the extracellular milieu via exosomes. More generally, in fluids or biopsies miRNAs exhibit very good stability over time independently of the technique of preservation (room temperature, freezing, paraffin fixation...). However, protocols still have to be standardized for the extraction of these miRNA and the use of their quantification for clinical purposes *(Gaarz and al., 2010; Leidinger and al., 2010; Kanke and al., 2009; Michael and al., 2010).* Nonetheless, the presence of oncomiR and the repression of tumor suppressor miRNAs indicate that modulation of their expression has a real therapeutic interest. In this view, many molecules have been synthesized in order to inhibit the expression of miRNAs with the use of specific antagonist such as antagomirs *(Krutzfeldt and al., 2005)*. An in vivo study of the specific inhibition of miR-122 in a model of hepatic metabolic disease was performed.

The intravenous injection of miR-122-specific antagomir in mice leads to a decrease in the miRNA expression in the liver correlated with a decrease of the cholesterol amount in the plasma of obese mice *(Esau and al., 2006)*. Moreover, other molecules, called miRNA mimics, have been developed to allow re-expression of tumor suppressor miRNAs. For example, the Let-7 family has been shown to be particularly down-regulated in many cancers (stomach, breast, pancreas, cancer). Trang et al. have used an in vivo KRAS dependent lung cancer model where they re-introduced the expression of Let-7a and b with specific mimic, which resulted in a decrease in tumor size *(Trang and al., 2011).*

However, the targeting of miRNAs in therapeutic approaches against cancer has limitations. Indeed, miRNAs have multiple target genes which results in a decrease of the specificity of action and an increase in the side effects development related to the treatment. Furthermore, methods for the *in vivo* delivery of antagomir and mimic molecules remain to be optimized. Currently, lipids or viral vectors are used. Unfortunately these techniques lead to DNA damage and strong immune response. In addition, the viral vectors are potentially dangerous when they integrate in the human genome *(MacFarlane and al., 2010)*. Slack and colleagues have shown that the use of nanoparticles for stabilized and packed inhibitors can be beneficial *(Babar and al., 2012; Kosinski and al., 2011)*.

4. Epigenetic aspects of Gastric Cancer

GC, like other cancers, presents a globally hypomethylated genome with some local hypermethylation *(Nursal and al., 2016)*. These epigenetic changes, once characterized, helped ranked GC from a molecular point of view. The TCGA define different molecular subtypes for GC, the EBV-positive tumors (EBV-GC), the microsatellite unstable tumors (MSI), the genome stability tumors (GS) and the chromosomal instability tumors (CIN). As described previously, EBV-GC and MSI subtypes are characterized by alterations in DNA methylation (***See molecular characterization of GC section***).

4.1. Histone modification: the HDAC

The enzymes allowing DNA and histones methylation have the ability to recruit HDACs to consolidate gene silencing. An alteration in expression of HDACs is observed in many cancers. However, few cases of aberrant HDAC expression have been described in the context of the CG. HDAC1 and HDAC2 are overexpressed in advanced types and their expressions are correlated with poor prognosis for patients *(Mutze and al., 2010)*. Moreover, HDAC1 is known to downregulate p53

and the von Hippen Lindau factor expression, which are both implicated in apoptosis and angiogenesis processes *(Choi and al., 2001)*. Furthermore, HDAC4 is overexpressed in GC cell lines leading to an increase in cell growth and apoptosis arrest *(Kang and al., 2014).*

4.2. Non-coding RNA

Recent studies showed the importance of miRNAs in the diagnosis and prognosis of cancers of the digestive system, but their precise role in this context is poorly known *(Zhang and al., 2008)*. For example, miR-21, who shows oncogenic activity (oncomiR), has been described to be overexpressed in 92% of GC, and correlated with inhibition of the expression of its target gene PTEN, a tumor suppressor. Moreover genomics loss of the tumor suppressor miR-101 results in the overexpression of EZH2 and concomitant deregulation of epigenetic pathway, which leads to cancer progression *(Ishiguro and al., 2014)*. Upregulated miR-106 and down regulated miR-203 are associated with gastric tumor size and, stage, as well as lymph nodes and distant metastasis. They may become new predictive markers in GC *(Xiao and al., 2009; Chiang and al., 2011)*. MiRNAs often organize in clusters and they share common functions. For example, miR-222-221 and miR-106b-25 which are known to be upregulated in GC tissues, leads to the activation of CDK2 activity to increased the transition in the G1/S phase *(Liao and al., 2011; Cho and al., 2009)*. MiRNAs are also related to the apoptosis process, as miR-375 described to suppress Akt phosphorylation pathway and inhibit 14-3-3ζ an apoptotic gene, which is down regulated in various GC types *(Tsukamoto and al., 2010)*. In addition, some of them are linked to the metastasis formation, as Let-7 family members which show a inverse relationship with their target gene HMGA2, implicated in gastric tumor invasion *(Motoyama and al., 2008)*.

Other miRNAs have been described to play a role in the chemosensitivity of the GC cells. For example, expression of miR-143, miR-144 and miR-145 is described as a prognosis marker of the chemotherapy effectiveness *(Takagi and al., 2009; Akiyoshi and al., 2012)*. Finally, the ectopic over expression of miR-15b and miR-16, which are down-regulated in multidrug resistant gastric cancer cell lines, leads to a chemo-sensitizing effect via Bcl2 inhibition, an anti-apoptotic gene *(Xia and al., 2008)*.

Many team attempted to define a miRNA expression signature in GC. The recurring problem of these studies is the general lack of criteria for selection of samples analyzed. Indeed, these retrospective studies do not often take account of the stage of the tumor, histological characteristics (intestinal, diffuse), and do not distinguish between the different treatments

administered to the patient. The results are biased and give rise to much controversy for the miRNA status: oncogenic or tumor suppressor. This is the case for the cluster miR-92/miR-182/ miR-183 overexpressed in GC but whose status is not clear. In fact Li et al. described it for its role in the initiation of metastatic development while Tang et al., gave it the opposite effect *(Zhang and al., 2013b)*.

IV. P53 FAMILY

1. p53 family members and gastric cancer

It has been reported that proteins of the p53 family (p53, p63, p73) are involved in many aspects of cancer development in the digestive system. p53 is mutated in 60% of GC. Expression of ΔNp73 isoforms correlates with poor prognosis for patients with GC *(Rivlin, and al., 2011)*. Moreover, p53 mutants interfere with the functions of TAp73 isoforms in GC cells in response to cytotoxic therapies *(Rufini and al., 2011)*, including platinum derivatives compounds. Inactivation of p53 in GC is one of the crucial steps leading to cancer progression and can be explained by genetic and epigenetic modifications. For example, p53 expression can be decreased by miR-125b controlling the balance between proliferation and apoptosis and enhancing cancer progression *(Rokavec and al., 2014)*. Furthermore, p53 can interact with HDAC1, which will reduce its binding capacity to *BAX* promoter, an apoptotic gene and improve survival of the cells *(Juan and al., 2000)*.

Moreover, I have presented throughout this work the involvement of the p53 members in the answer to some common (cisplatin) or in development (RDC11) chemotherapies. Cisplatin and RDC11 have the ability to induce the expression of p53 and p73, thus allowing apoptosis of tumor cells. These observations have led us to further study the p53 family members, mainly p53 and p73 in the context of GC.

2. Generality about the p53 family

The p53 family is composed of 3 members: p53, p63 and p73. These transcription factors play a key role in many biological processes such as cell cycle arrest, DNA repair, senescence and apoptosis.

p53 was first described in 1979. It is defined as a "guardian of the genome" (Lane, 1992) or as a "cellular gatekeeper" (Levine, 1997). Its main functions are related to cell cycle arrest and the induction of apoptosis. Moreover, it also plays an important protective role against the development of cancer. Mice knockout for p53 are viable without major development defect but are more likely to develop spontaneous tumors *(Donehower and al., 1992)*. In the same context, the inherited germline mutation of p53, found in the Li-Fraumeni syndrome, predisposes patients to cancer development such as breast cancer *(Rivlin and al., 2011)*. The p53 activity is also critical in response to chemotherapy. Thus, its inactivation by deletion, mutation or sequestration by viral proteins is a key step in the development of over half of the human cancers.

Several years after the discovery of p53, p63 and p73 have been described *(Yang and al., 1998; Kaghad and al., 1997)*. They show a high sequence similarity to p53 but their function seems to be more important in the developmental process. Indeed the generation of knockout mice for p63 and p73 have been achieved, and in contrast to p53, they do not appear to develop spontaneous tumors. The p63-deficient mice are viable but because of the major development defects, observed, they die shortly after birth. They present a total loss of stratified epithelium and glandular structures (thymus, prostate…) *(Mills and al., 1999; Rouleau and al., 2011)*. These observations highlight the importance of p63 in the development of the epithelia. Mice deficient in p73 have a high mortality rate after birth. They develop major gastrointestinal and intracranial haemorrhages. Death occurs 4-6 weeks after birth due to chronic infections. Their phenotype is characterised by the presence of hydrocephalus, hippocampal dysgenesis, and default in pheromones detection, which is crucial for social behavior. This phenotype suggests an important role of p73 in neurogenesis *(Yang and al., 2000).*

3. Domains organization

p63 and p73 have a domain organization similar to p53, with the presence of three functional domains: an amino-terminal transactivation domain (TAD), a DNA binding domain (DBD) and an oligomerization domain (OD). In contrast to p53, p63 and p73 have a long carboxyl terminus containing a sterile alpha domain (SAM) involved predominantly in the developmental regulation by protein-protein interactions. The presence of this domain supports the fact that these two members play a key role in the process of cell differentiation *(Candi and al., 2013)* **(Figure 21)**.
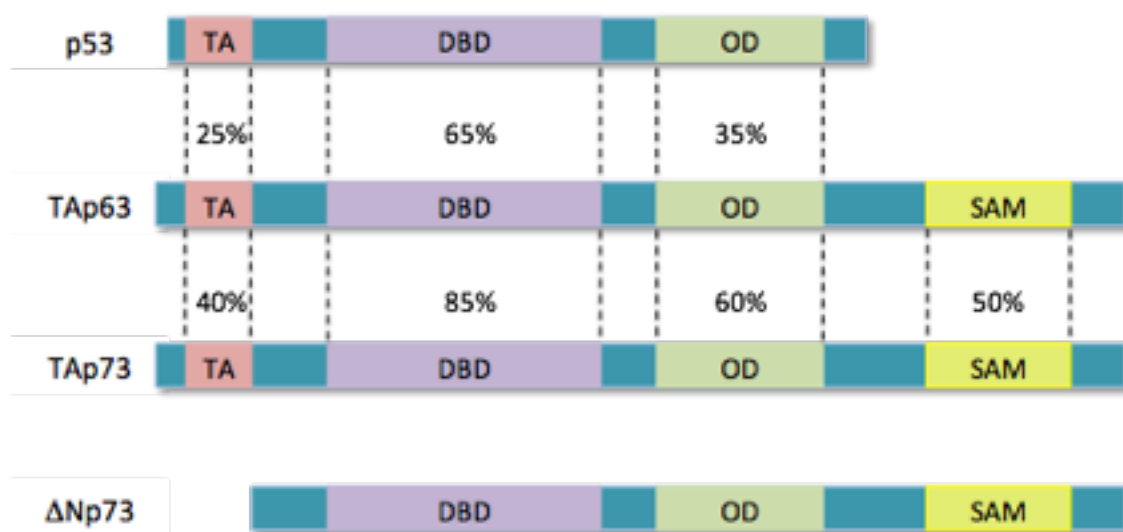

Figure 21: Structure of the p53 family members

DNA-binding domain (DBD)

The three members have a high sequence homology in their DBD (63% between p53 and p73 and 60% between p63 and p53). The DBD gives them the ability to bind to specific sequences in the promoters of genes involved in cell cycle arrest (p21, GADD45) and apoptosis (Bax, PUMA) *(Harms and al., 2004)*. The sequence homology between these DBDs confers to them the ability to transactivate the expression of the same genes but also to regulate the expression of each other *(Stiewe, 2007)*. Indeed, TAp73 and p53 can activate the transcription of ΔNp73. Conversely, ΔNp73 is able to bind to the p53 response element (p53 RE) on the TAp73 and p53 promoter inducing their downregulation. In addition to these shared target genes, each of them also have specific ones *(Stiewe and al., 2002)*.

Oligomerization domain (OD)

Members of the p53 family bind as tetramers to the DNA by their OD present in the C-terminal region. The mutation of the OD leads to the inhibition of p53 activity, confirming the fact that their activity is dependent of the tetramer formation *(Davidson and al.,2001)*. Wild type p53 does not seem to form heterotetramer unlike p63 and p73 *(Davidson and al., 1999; Coutandin and al., 2009)*. However, p53 mutants can oligomerise with all the family members, as well as ΔNp63 and ΔNp73, leading to the inhibition of p53, TAp73 and TAp63 activity *(Gaiddon and al., 2001).*

Transactivation domain (TAD)

Each family member has two different promoters: one upstream of the first exon (the distal promoter, P1) and one located within intron 3 (the internal promoter, P2) for the generation of different isoforms. Thus, transcription from P1 generates p53, TAp63 and TAp73, each with a transcriptional activity. The loss of the TAD due to transcription initiated in P2, leads to the formation of the isoforms called « ΔN », ΔNp63 and ΔNp73. The ΔN isoform, also called the dominant negative, lacks is transcriptional activity without the TAD. They can still bind to DNA but cannot interact with the transcriptional machinery. For this they act by competition with the transactivated isoforms *(Yang and al., 2002b)*. For example, ΔNp73 is able to bind on the p53 and TAp73 target gene promoters to inhibit their transcription *(Grob and al., 2001)*. However, recent studies suggest that the ΔNp63 and ΔNp73 isoforms could still have a transcriptional activity by the presence of small non-conventional TAD domain in the N-terminal extremity *(Lin and al., 2009)*.

Originally, only p63 and p73 were described for the generation of different N-terminal isoforms. Recently, p53 was reported to also have an intronic promoter (Δ133p53, Δ160p53). Furthermore,

alternative splicing of p73 and p53 is observed generating unconventional TA isoforms (ΔEx2p73, Δ40p53) *(Murray-Zmijewski and al., 2006; Marcel and al., 2011; Wei and al., 2012)*.

Carboxi-terminal domain (CTD)

Alternative splicing in the CTD of p53, p63 and p73 is observed, generating different transcriptional variants. For p53, 7 variants are described (α, β, γ, δ, ε, ζ, ΔE6p53) *(Bourdon and al., 2005; Hofstetter and al., 2010)*. The p63 gene was shown to express at least 5 alternatively spliced C-terminal isoforms (α - ε), while the p73 gene expresses at least 7 alternatively spliced C-terminal isoforms (α - η) *(Mangiulli and al., 2009; Wei and al., 2012)*. Therefore, the p63 gene expresses 6 mRNA variants which code for 6 different p63 protein isoforms, while p73 gene expresses at least 35 mRNA variants which code for 29 different p73 protein isoforms. Currently no animal model has been conducted to determine what the functions of these isoforms are. The majority of studies was performed on the α and β isoforms and show major differences. For example, TAp73β seems to mimic the effects of p53 through the transcriptional activation p53 target genes involved in apoptosis. Conversely, TAp73α is involved in gene regulation implicated in metastasis-related function as the focal adhesion and the actin cytoskeleton. In addition, the function of these isoforms would be dependent on the cell type and the cellular context *(Logotheti and al., 2013)*.

Differences in the sequence and the structure of these three members lead to some differences in the protein-protein interactions, differences in post-translational modifications and particularly differences in transcriptional activation of target genes. Now, I will develop in more details the major function of p53 and p73, especially in the context of GC.

4. Functions of p53

p53 has been described to regulate several biological processes such as cell cycle progression, senescence, DNA metabolism, angiogenesis, cell differentiation, immune response and apoptosis. However, its major functions are involved in growth arrest and apoptosis.

4.1. p53 regulation

p53/MDM2 repressor complex

p53 is a sensor of DNA damage and genotoxic stress. Thus, under normal conditions, p53 is expressed at low levels in the cell, through its interaction with MDM2 protein, an E3 ubiquitin ligase. The ubiquitinylation of p53 allows its proteasomal degradation *(Toledo and al., 2006)*. Knockout of MDM2 causes embryonic lethality in mouse, due to the aberrant expression of p53

(Marabese and al., 2007). In contrast, p53 is itself able to regulate the expression of MDM2 as part of a negative feedback loop. In addition, MDM2 is able to process to its auto-ubiquitinylation allowing the disruption of the p53-MDM2 complex *(Honda and al., 1997)*. In response to DNA damage or genotoxic stress, Kruse and Gu proposed a model in 3 steps for the activation of p53 target promoters *(Zilfou and al., 2009)*:

- p53 stabilization in response to cellular stress through the inhibition of MDM2
- p53 derepression by post-translational modifications
- cofactors recruitments for transcriptional activation of p53 target promoters

p53 post-translational modifications

p53 can be phosphorylated allowing in one hand to increase the selectivity of its target genes and on the other hand the inhibition of the MDM2 complex formation *(Bode and al., 2004)*. P53 sequence selectivity is increased in response to DNA damage by the action of CK1 (casein kinase 1), which phosphorylates the serine (Ser) 6-9 and threonine (Thr) 18 residues. γ-irradiation will allow to activate ATM, a sensor of DNA damages, which will be able to hyperphosphorylate MDM2 and allow the p53 release *(Shiloh and al., 2001)*. Moreover, ARF and INK4, important in cell cycle progression and regulation of p53, have the ability to inhibit the complex p53 / MDM2 formation through MDM2 sumoylation *(Xirodimas and al., 2002)*.

p53 acetylation prevents its ubiquitination and therefore its degradation by the proteasome. For example, the acetyltransferases CBP (p300/CREB) and PCAF (p300/CREB associated factor) coactivate p53 by increasing its stability and transcriptional activity. To respond to p53 activation, the target gene promoters must be accessible. By interaction with p300/CBP, p53 promotes the histone acetylation around its response elements, thus inducing chromatin relaxation *(Chan and La Thongue, 2001)*. Conversely, the action of histone deacetylases, such as HDAC1, inhibits p53 activity *(Juan et al., 2000; Luo et al., 2000)* promoting its ubiquitin-dependent degradation.

However, post-translational modifications of p53 can also induce its MDM2-independent degradation by JNK, which plays a dual role. Under normal conditions, JNK (c-Jun N-terminal kinase) interacts with p53 during the G0/G1 transition of the cell cycle and induces its ubiquitination and degradation *(Buschmann and al., 2001; Fuchs and al., 1998)*. Conversely, in response to DNA damage, JNK induces p53 phosphorylation and activation. p53 can also be neddylated by NEDD8 mediated by MDM2 which causes its destabilisation and thus its degradation *(Xirodimas and al., 2004)*. Moreover, Katayama and colleagues showed that the aurora kinase A, which is overexpressed in bladder cancer had the ability to phosphorylate p53 (Ser315, C-

terminal), which induces its destabilisation and therefore its degradation *(Katayama and al., 2004)* .

In response to DNA damages, p53 is involved in the cell cycle to prevent DNA replication and genetically altered cell division, proof of its importance in the maintenance of the genetic integrity. It can promote DNA repair, cell senescence or apoptosis (Zilfou and al., 2009). How p53 chooses its program is not clear yet. Indeed, this choice could firstly be dependent of the post-translational modifications but also to the damage extent and the stress duration, as proposed by Vousden and Prives *(Vousden and Prives, 2009)* (**figure 22**).
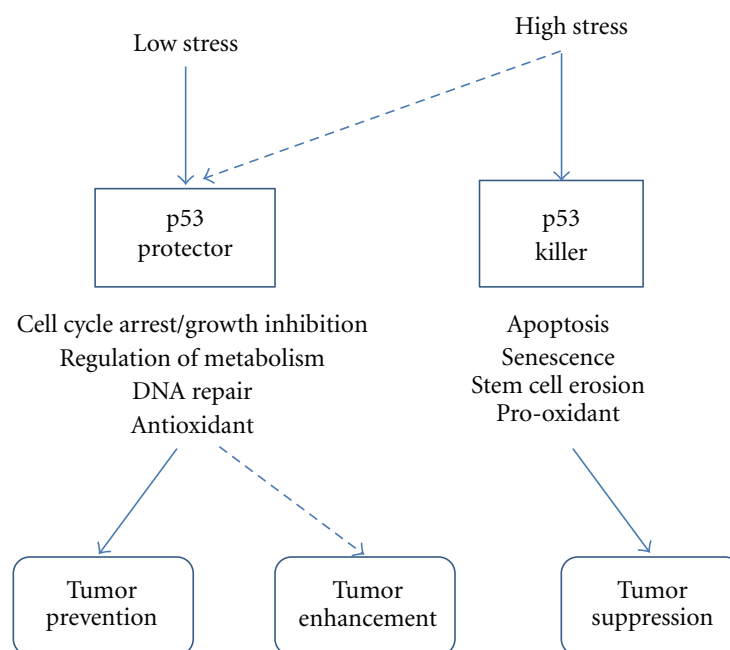
Figure 22: Vousden and Prives [2] proposed a model of the dual mechanism of p53 function in tumors. *Suzuki and al., 2011*

4.2. Cell cycle arrest

The cell cycle progression is regulated by the combination of cyclin and cyclin-dependent kinase (CDK) *(Nurse 2000)*. In response to UV, p53 has the ability to arrest the cell cycle in the G1 phase by activating the transcription of one of its target genes, p21 (a cyclin-dependent kinase inhibitor) that blocks the cell cycle by interacting with and inhibiting CDK1, 2, 4 and 6 *(Gartel and al., 2005)*. p53 may also arrest the cell cycle in the G2 / M transition through the maturating-promoting factor (MPF) composed of the cyclin B1 / cdc2 complex *(St Clair and al., 2006)*. Indeed, p53 activates

transcription of the 14-3-3Ơ factor that prevents the proper nuclear localization of the cyclin B1 / cdc2 complex and therefore inhibits its activity *(Chan and al., 1999)*.

Following the cell cycle arrest, p53 can induce DNA repair. GADD45a activated by p53 is involved in this process through its interaction with the histone, creating a more accessible chromatin conformation *(Zhan and al., 1999)*. For example, in response to cisplatin, p53 has the ability to activate the transcription of XPE and XPC who will recognize DNA adducts and allow the DNA repair by the NER *(Shimizu and al., 2003; Fitch and al., 2003)*. In addition, in response to DNA damages, p53 activates the transcription of MSH2, a protein of the MMR in order to improve its effect and to reduce the microsatellite instability.

Activation of the senescence, the permanent arrest of cell division, may also promote p53 through p15, p16 and p21. p15 and p16, unlike p21 bind permanently CDK4 and CDK6, thereby preventing the recovery of the cell cycle. This mechanism is particularly advantageous in inhibiting tumor growth *(Serrano and al., 1997)*. Xue and al used an animal model of liver cancer. When they reactivated p53 expression they showed that p53 allowed a decrease of the tumor size by the introduction of the cell senescence followed by an immune system response leading to the tumor cell death *(Xue and al., 2007; Bertheau et al., 2008).*

4.3. Apoptosis

This mechanism is particularly important in the inhibition of tumor development. p53 is a major actor of this process, which is emphasized by the fact that p53 is mutated or deleted in over half of human cancers *(Murray-Zmijewski and al., 2006)*. In addition, apoptosis is lost in p53 deficient tumors *(Parant and al., 2003)*. The two apoptotic pathways, the intrinsic and extrinsic pathway can be modulated by p53 (**Figure 23**).

The extrinsic pathway of apoptosis

This pathway involves the cell surface receptors of death domain, Fas and DR5, inducing the apoptosis following an external stimulus (inflammation). The binding of their ligand (Fas ligand or TNF) induces the capacity of the death receptors to bind to proteins adapter and activate the pro-Caspase 8 cleavage, which itself activates caspase 3 leading to the apoptosis *(Thornberry and al., 1998)*. Caspase 8 also has the ability to communicate with the intrinsic pathway by the cleavage and activation of Bid, a pro-apoptotic Bcl-2 family member who participates in the release of the cytochrome c from the mitochondria by activation of Bax *(Tait and al., 2010)*. p53 is involved in this

pathway by its ability to activate Fas and DR5 receptors and the Caspase 8 expression *(Fukasawa and al., 1999; Wu and al., 1997; Liedtke and al., 2003)*.
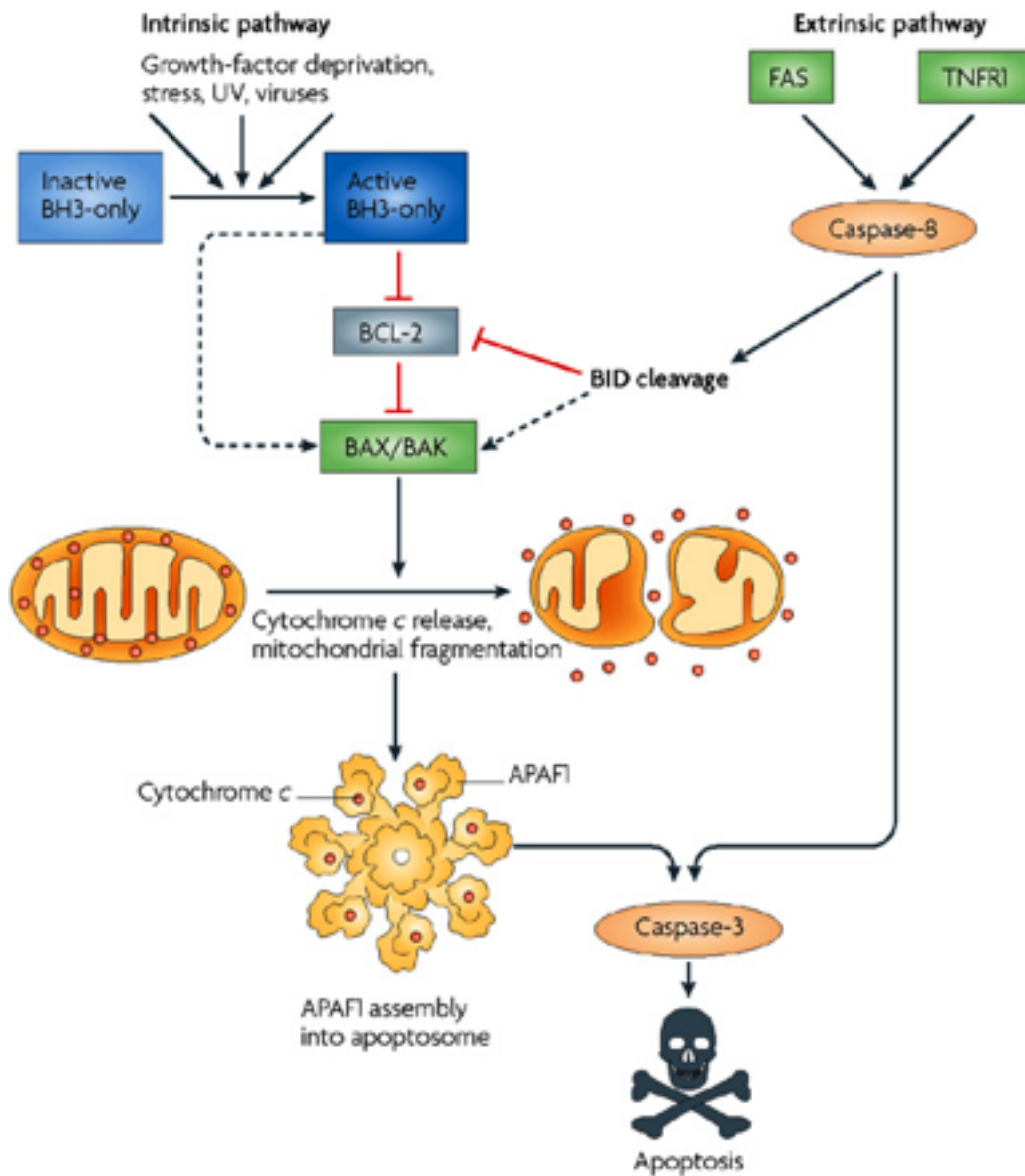
Figure 23: Scheme depicting intrinsic and extrinsic pathways of apoptosis. *Youle and al., 2008*

The intrinsic pathway of apoptosis

This pathway is connected to the activity of the mitochondria. p53 will allow the transcriptional activation of several pro-apoptotic genes leading to an increase in the expression of the BH3-only proteins, Puma and Noxa and the Bcl-2 family proteins, Bax and Bid *(Fridman and al., 2003)*. Puma-deficient mice present a phenotype similar to that observed with p53 -/- in the absence of the apoptosis implementation *(Suzuki and al., 2011)*. Bid activates Bax, while Noxa and Puma bind the anti-apoptotic Bcl-2-family proteins, Bcl-2 and Bcl-xL, inhibiting their activity. In addition, the Cytochrome c is released from the inner membrane of the mitochondria, and associates with

APAF1 and Caspase 9 to form the apoptosome. This complex will then activate the Caspase 3 among others, which allows the DNA condensation and fragmentation, the proteolysis, the compaction of the cell membrane and budding. The cell membrane disrupts the cellular components to encapsulate them before being cleared by macrophages *(Elmore, 2007; Hail and al., 2006).*

5. p73 in tumorigenesis

Originally, p73 has been described for its role in neuronal development after the generation of p73 knockout mice *(Mills and al., 1999; Yand and al., 2000)*. These models do not seem likely to develop spontaneous tumors such as p53 -/- mice. Indeed, the knockout mice were deficient for all the p73 isoforms, TAp73 and ΔNp73. Flores and colleagues first showed that the generation of mice heterozygous for p73 developed cancers such as lung, squamous cell cancer and thymic lymphoma *(Flores and al., 2005)*. Thereafter, Tomasini and al. and Wilhelm and al. generated mice selectively inactivated for TAp73 and ΔNp73 respectively. TAp73 deficient mice develop spontaneous tumors, as observed with p53. Conversely, ΔNp73 deficient mice do not have this characteristic. Embryonic fibroblasts derived from those mice exhibit an up regulation of the p53 family target genes, *CDKN1A* (p21), *PUMA, MDM2*, suggesting that in normal cells, ΔNp73 represses transcription of these genes. These experiments highlight the tumor suppressor activity of the TAp73 isoform and the oncogenic activity of ΔNp73. *(Tomasini and al., 2008; Wilhelm and al., 2010)*.

Surprisingly, p73 is rarely mutated in human cancers (less than 0.5%), in contrast to p53. Conversely, many cancers, such as neuroblastoma, stomach, breast and colon cancer, exhibit an aberrant p73 expression *(Moll and al., 2001)*. However, we should be careful with these results because many of them do not take into account the ratio of TAp73/ΔNp73 expression, consequence of a lack in the technical detection. Casciano et al. showed that ΔNp73 expression in neuroblastoma correlated with decreased survival and poor outcome for the patient regardless of age, primary tumor site, and localisation *(Casciano and al., 2002)*. Like p53, p73 is expressed at low levels in cells in normal conditions and activated in response to DNA damages. Its activation is modulated by some transcriptional and post-translational modifications enabling it to have a role in various biological processes similar to p53.

5.1. p73 regulation

The question that remains unclear is why the cells favor in some case TAp73 or ΔNp73 transcription. The balance between these two isoforms could be broken by the change in the initiation of the transcription at the P1 and P2 promoters, by a difference in mRNA stability or the change of the protein status through post-translational modifications *(Marabese and al., 2007).*

Transcriptional regulation

The p73 promoter contains response elements for diverse transcription factors that control its transcription, such as E2F1. E2F1 is involved in the regulation of various genes involved in the cell cycle, the cell fate, the DNA damage repair and the apoptosis. In response to DNA damage, the repressor complex C-EBPα / E2F1 is disrupted by the acetylation of E2F1 for the C-EBPα translocation out of the nucleus and the activation of E2F1 *(La Thangue and al., 2003)*. E2F1 then has the ability to initiate the apoptosis in the cells by the activation of p73 and some pro-apoptotic target genes of p53 *(Irwin and al., 2000)*. Moreover, p53 itself can activate the expression of p73 *(Chen and al., 2001)*. Conversely, the transcription of the p73 gene may be also inhibited by certain factors such as ZEB1 and ΔNp73. Regulation by ZEB1 is linked to the status of BRCA1 in breast cancer. Indeed BRCA1 negative cells have a p73-dependent apoptosis in response to cisplatin, whereas it is negligible when BRCA1 is present. Indeed, ZEB1 is repressed when BRCA1 is absent because of its methylated promoter *(Fontemaggi and al., 2001)*. ΔNp73 can also activate p53 and TAp73 highlighting the presence of a negative feedback loop *(Grob and al., 2001)*.

Post-translational regulation

p73 is regulated by some post-translational modifications (ubiquitination, sumoylation, acetylation) leading to the degradation or stabilisation of TAp73 and ΔNp73 isoforms.

MDM2 also has the ability to interact with p73. However, the formation of this complex does not induce the degradation of p73, but rather an increase in its stability in contrast to what is observed with p53 *(Leng and al., 2003)*. Similarly, SUMO1, an ubiquitin-like protein, will modulate the interaction of p73 with other factors such as c-Abl and increase its stability *(Minty and al., 2000)*. Conversely, ITCH, a Hect ubiquitin ligase, and NQO1 (ubiquitin-independent/20S proteasome), will induce the p73 proteasomal degradation in absence of stress in the cell *(Rossi and al., 2005b)*. Finally, UFD2a (U-box type E4/E4 ubiquitin ligase) binds p73α at its SAM domain. In response to cisplatin, the expression of UFD2a is reduced in neuroblastoma cells, thereby inducing the activation of p73 and the apoptosis *(Hosoda and al., 2005)*.

The expression of p73 may be modulated without affecting its protein level. For example, PIAS1 allows TAp73 sumoylation, which causes a decrease in its transcriptional activity at the G1 phase of the cell cycle *(Munarriz and al., 2004)*. The acetylation of p73 by the p300/PCAF complex increases its activity, unlike the action of SIRT1 (a histone deacetylase), which inhibits its activity *(Dai and al., 2007)*. In response to genotoxic stress, phosphorylation of p73 by c-Abl, a tyrosine kinase, p38 / MAPK, and Chk1 increases its stability and activity *(Urist and al., 2004a)*. The p73-dependent apoptosis cannot be induced if the cells are deficient for c-Abl. Conversely, its phosphorylation by the cyclin / CDK complex inhibits its activity thereby permitting cell cycle progression *(Rufini and al., 2011)*.

Promoter binding affinity regulations

The combination of post-translational modifications allow p73 activation in response to DNA damage, but also its interaction with certain proteins that have the ability to change its binding affinity to the promoter of its target genes. For example, ASPP1 and ASPP2 interact with p73 to promote the transcriptional activation of pro-apoptotic genes *(Robinson and al., 2008; Patel and al., 2008).*

p53 mutants (143A, 175H, 173L) have the ability to bind to p73 inhibiting its transcriptional activity. Bruno and al. showed that the transcription regulation of these mutants is dependent of Che1, a RNA Pol II-binding-protein. Inhibition of Che1 expression in cancer cell lines enables the decrease of the p53 mutants and DNA repair proteins expression. This results in the S phase arrest, and in the accumulation of DNA damage. This leads to the activation of p73 modulated by E2F1 to induce apoptosis *(Bruno and al., 2010).*

Finally, EBV infection, found in 10% of gastric cancer was correlated with the inhibition of p73 activity. Indeed, the expression of the EBV nuclear antigen EBNA3C regulates cell cycle progression through its interaction with p73 but also p53. This induces the development of chemotherapy resistance (doxorubicin in the case of lymphoma) because of the TAp73 activity inhibition and ΔNp73 stabilization. Through its interaction with TAp73, the viral protein is able to inhibit protein-protein interaction with other pro-apoptotic factors. In addition, EBV is also responsible for the genomic instability associated with loss of expression of p73 by promoting the promoter hypermethylation through DNMT *(Sahu and al., 2014).*

Following its activation, TAp73 is able to mimic the p53 functions through its role in cell cycle arrest and apoptosis.

5.2. Role in the cell cycle

TAp73 has been shown to play a role in the regulation of mitosis and in the maintenance of genomic stability. Aneuploid and polyploid cells are found in tumors. This is a necessary mechanism to tumor development, generating a genomic instability. TAp73 knockdown mice show the presence of primary polyploid cells following the aberrant activation of the cyclin / CDK complex during the S phase. In fact, TAp73 is located in mitotic spindle and interacts with the spindle assembly checkpoint (SAC) proteins (Bub1, 3) allowing their activation. Therefore it has a role in the sensing of improper attachment of sister chromatin to the mitotic spindle and delay the anaphase until all chromosomes are ready to segregate. In the case of a prolonged mitosis arrest or a failure of the SAC, TAp73 has the ability to induce cell apoptosis *(Tomasini and al., 2008; Tomasini and al., 2009; Vernole and al., 2009).*

TAp73 also allows the cell cycle arrest in G1 in response to DNA damage by the transcriptional activation of p21 and p57. Conversely, ΔNp73 is able to inhibit their expressions by competing with TAp73 on their response elements or by its oligomerization with TAp73, inhibiting its activity. Like p53, it can also stop the cycle in the G2/M transition by inhibiting the activity of different factors such as the Cyclin B1 / cdc2 complex *(Allocati and al., 2012)*. Moreover TAp73 prevents replicative immortality. Telomeres represent the guardians of the chromosomal integrity. Telomeres length is reduced each cell cycle, and apoptosis of the cell is induced when they become too short. An increase in the hTERT telomerase expression, modulated by SP1, is observed in cancers to prevent apoptosis. TAp73 has the ability to inhibit the transcriptional activation of hTERT by competing with SP1 *(Hanahan and al., 2011; Beitzinger and al., 2006)*. Conversely, ΔNp73 allows hTERT induction by disrupting the formation of the E2F/Rb complex *(Racek and al., 2005)*. Additionally, through its direct interaction with Rb, ΔNp73 leads to its hyperphosphorylation and thus the cell cycle progression *(Stiewe and al., 2003)*.

5.3. Apoptosis

The generation of heterozygous model for p73 and p53 shows a more aggressive tumor phenotype that be one observed with mice heterozygous for p53 alone *(Irwin and al., 2003; Flores and al., 2005)*. These observations tend to assume that p53 requires the p73 activity to induce apoptosis, unlike p73 who has the ability to play its role of tumor suppressor in absence of p53. These results are supported by the switch for the p73 expression and the initiation of apoptosis observed in a model of AML and breast cancer in a prolonged lack of p53 *(Chakraborty and al., 2010)*.

Apoptosis induced by p73 requires the presence of MLH1, a MMR protein, and c-Abl in response to cisplatin. This has been demonstrated in colon cancer cells and in mouse embryonic fibroblasts (MEF) deficient for the two factors. In response to ionizing radiation and DNA damaging agents, c-Abl will be activated by the MMR and interact with p73 and p300 *(Gong and al., 1999; Yuan and al., 1999; Agami and al., 1999)*. The formation of this complex is promoted by the activity of YAP1 (Yes Associated Protein) and Pin1 (prolyl Isomerase) and its interaction with PMS2 (MMR protein) *(Lapi and al., 2008; Urist and al., 2004b; Mantovani and al., 2004)*. c-Abl will allow the phosphorylation and the accumulation of p73. Nozell et al. showed that c-Abl was necessary for the acetylation of p73 by the p300 / PCAF complex *(Nozell and al., 2003)*. The acetylation of target genes involved in cell cycle arrest (p21, p57) mediated by p300 is not required in contrast to the pro-apoptotic genes (*BAX*) *(Costanzo and al., 2002; Zeng and al., 2001)*. This is followed by the induction of the intrinsic apoptotic pathway through the activation of Bax and Puma. Moreover, as p53, p73 is able to regulate the activity of the death receptors involved in the extrinsic apoptotic pathway.

Conversely, ΔNp73 promotes cell survival, but also modulates the chemosensitivity of the cell. Müller and al describe that the anti-apoptotic effect of ΔNp73 takes effect at different stages of apoptosis, from the death receptor to the mitochondria. It is important to define the ratio of TAp73/ΔNp73 to predict the clinical response following chemotherapy *(Müller and al., 2005)*.

6. p53 family members and HDAC

As I already described, p53 stability and activity are dependent on post-translational modifications such as acetylation. p53 was the first non-histone substrate described to be target by the HDAC *(Gu and al., 1997)*. In this context, p53 inactivation, frequently found in many cancers can be the result of its deacetylation by the HDAC enzymes promoting tumorigenesis. In fact, HDAC can directly or indirectly modulate p53 expression and activity depending on the cellular context. Furthermore, upon DNA damage, p53 itself has the ability to recruit HDAC through its interaction with the the co-repressor complex mSin3A for the repression of p53 target genes, such as *MAP4* and *STATHMIN*, two anti-apoptotic factors *(Murphy and al., 1999)*.

6.1. Consequences of p53 deacetylation

p53 deacetylation can leads to its degradation through the MDM2 / HDAC co-repressor complex formation. The acetylation of p53 prevents its degradation by MDM2: acetylated residues correspond to those normally ubiquitinylated *(Chan and La Thongue, 2001; Brooks and al., 2011)*.

Ito and al show that MDM2 is able to inhibit p53 acetylation through its binding to the acetyltransferases p300/CBP, reducing their activity and thereby inducing p53 proteasomal degradation. However, HDACi treatment restores p53 acetylation highlighting HDAC implication in this mechanism *(Ito and al., 2001)*. Indeed, MDM2 has the ability to recruit HDAC1 allowing p53 deacetylation and ubiquitination. Thus, ectopic expression of a dominant negative mutant of HDAC1 restores p53 acetylation *(Ito and al., 2002).*

p53 acetylation induces also the conformational change of its DBD and therefore its sequence specific recognition and transcriptional activity *(Hupp and al., 1995)*. p53 deacetylation by HDAC1 reduces its ability to activate its target genes such as *BAX*, an anti-apoptotic gene *(Juan and al., 2000)*. In parallel to the discovery of Juan and his colleagues, Luo and al. described that p53 deacetylation by HDAC1 is the result of its interaction with the co-repressor complex NuRD, reducing p53 ability to induce cell cycle arrest and apoptosis *(Luo and al. 2000)*.

6.2. Indirect modulations of p53 / p73 activity by the HDAC

The transcriptional activity of p53 may be modulated by HDACs regardless of its acetylation level. Harms and al showed that HDAC2 knockdown in breast cancer cells induced a cell cycle arrest in G1 phase, a reduction in cell proliferation and the development of cell senescence via p53. Deletion of HDAC2 leads to a higher trans-repression (*MYC*, *CCNB1*) and trans-activation (*CDKN1A*) of p53 target genes. HDAC2 however has no effect on the acetylation level of p53 but on its ability to bind to DNA. Through its ability to interact with the topoisomerase II, HDAC2 could modulate the chromatin structure which will affect p53/DNA binding activity *(Harms and al., 2007)*.

p53 mutants are found in more than 50% of cancers and are implicated in resistance mechanisms to chemotherapy. HDAC8 has the ability to upregulate the transcriptional expression of mutant p53 via HOXA5. HOXA5 is a known transcriptional factor implicated in the positive regulation of p53 transcription. Indeed, HDAC8 knockdown causes the reduction of cell proliferation and the induction of cell cycle arrest in colon cancer cells. The mechanism involved in the regulation of HOXA5 by HDAC8 is not yet known *(Yan and al., 2013)*. Furthermore, HDAC6 has been described to be a positive regulator of HSP90 activity required for its management of many oncogenes such as p53 mutants. The knockdown of HDAC6 leads to the acetylation of HSP90 and thus the degradation of its client proteins *(Li and al., 2011; Hong and al., 2014)*.

Interestingly, Zhang and al. described HDAC1 as a key regulator of p73 stabilization. HDAC1 knockdown leads to the decrease in TAp73 half-life in normal and DNA damage conditions. Loss of HDAC1 expression results in the hyperacetylation of HSP90, making it inactive. HSP90 can no longer interact and stabilize TAp73 leading to its proteasomal degradation *(Zhang and al., 2013c)*. These experiments show a tumor suppressor and oncogenic activity of HDAC and Hsp90.

7. p53 family members and miRNA pathway

The presence of p53 mutants in gastric tumors results in the decrease of a subset of miRNA. For example, miR-34a, a really well known target of p53, is frequently downregulated in GC. It was shown to decrease migration and invasion by inhibiting the EMT pathway and enhancing apoptosis. It regulates this process by suppressing SNAIL and Bcl2 expression. Restoration of its expression inhibits the tumorspheres formation in gastric cancer cell lines and sensitized them to current chemotherapies like cisplatin *(Ji, Q. and al., 2008; Ji, Q. and al., 2009)*.

7.1. p53 - miRNA feedback loop

As I have already described, miRNA with a tumor suppressor activity can be activated by some transcription factors such as p53. Inactivation of p53 in many cancers results in a loss of miRNA expression with growth suppressive roles *(Jansson and al., 2012; Rokavec and al., 2014)*. Many miRNA are under direct or indirect control of p53.

Analysis of the miRNA expression profile in response to p53 activation through genotoxic stress allowed to highlight the family members miR-34 (a, b and c) as the most highly regulated by p53 *(Chang and al., 2007; He and al., 2007; Tarasov and al., 2007)*. Transcription of their pri-miRNA is under the control of p53. The members of the miR-34 family have the ability to target mRNAs expression involved in cell cycle regulation (CDK4/6, cdc25C) but also oncogenes (Myc, Bcl2) *(Hermeking, 2012)*. In view of the signaling pathways regulated by these miRNAs, we can conclude that miR-34 is an important relay in the establishment of the negative tumor growth regulation *(Raver-Shapira and al., 2007; Tarawa and al., 2007)*.Other miRNAs have been described to be regulated by p53. For example, miR-107, miR-200 and miR-192 inhibit angiogenesis and EMT *(Chang and al., 2011; Yamaguchi and al., 2010)*.

Furthermore, p53 is able to activate transcription of certain miRNAs known to target the expression of its repressor MDM2 (miR-194, miR-215) *(Pichiorri and al., 2010; Xiao and al., 2011)*. p53 can also suppress directly the activity of some oncomiR (miR-224, miR-502, miR-17-92 cluster).

The miR-17-92 cluster expression is altered in many cancers such as in colon cancer. They are involved in genomic amplification process and promote proliferation, survival, and tumor angiogenesis. Yan and al. showed that expression of this cluster was decreased in response to hypoxia by a direct p53 repression *(Yan and al., 2009)*.

Conversely, some miRNA may regulate the expression of p53 inducing its translational repression. miR-125b was the first described for its role as a p53 repressor by Le and colleagues *(Le and al., 2009)*. Furthermore miR-125b has the capacity to regulate p53 target genes, such as Puma and CDC25C. Through its activity, it can regulate the balance of genes involved in the arrest of proliferation and in the apoptosis. Its expression is altered in many cancers like in colorectal cancer and it is correlated with the tumor size, the invasive potential of the tumor and the poor prognosis for patients *(Nishida and al., 2011; Le and al., 2011)*.

Finally, p53 plays an important role in the regulation of miRNA biogenesis. For example, p53 has the ability to interact with p68 (a RNA helicase). p68 forms a complex with Drosha to promote specific miRNA processing *(Suzuki and al., 2009)*. Furthermore, a fine feedback loop between p53 and Dicer is described by Boominathan. p53 has the ability to modulate Dicer expression through the activation of certain miRNAs (miR-15/16a, miR-206, miR-103), thus promoting the maturation of miRNA involved in the inhibition of growth and induction of apoptosis *(Boominathan and al., 2010)*. However, the knockout of Dicer leads to the activation of p53 to induce an arrest of proliferation and the development of senescence. p53 has a role of checkpoint to monitor the miRNA process *(Mudhasani and al., 2008)*. Finally, Dicer is known to be regulated by p63, which may itself be altered by its oligomerization with the mutant p53 *(Su and al., 2010)*.

7.2. p73 - miRNA feedback loop

Involvement of p73 in the regulation of miRNA has been described for the first time by Sompath and colleagues in 2005. They have shown that the treatment of chronic lymphocytic leukemia (CLL) by HDAC inhibitors induces a p73-dependent apoptosis. Indeed treatment with a HDACi allowed the rise of repression for miR-106b. This miRNA has the ability to induce the degradation of ITCH, responsible for the degradation of p73, and thus to enhance the intrinsic apoptosis pathway *(Rossi and al., 2009; Sampath and al., 2009; Rivet di Val Cerno and al., 2009)*. Thereafter, TAp73 and p21 have been described in the transcriptional activation of miR-200c for its inhibition of ZEB1. ZEB1 is involved in the EMT and the metastatic development, but above all it is able to inhibit TAp73 expression *(Shimono and al., 2009; Burk and al., 2008)*.

TAp73 induces the expression of the Let-7 family members, known for their tumor suppressor activity. The Let-7 members can trigger Ago2 activation to favor their own process. However the processing of Let-7 may be inhibited by the induction of Lin28b through c-Myc. Therefore, p53 and TAp73 are able to reactivate the processing of Let-7 due to the induction of miR-145 which is able to target c-Myc and thus allows the EMT and tumor invasion inhibition *(Schwamborn and al., 2009; Boominathan and al., 2010; Ryback and al., 2008; Viswanathan and al., 2008).*

With bioinformatic analysis, Boominathan hypothesized diverse mechanisms for p73 to regulate the biogenesis of miRNAs. Indeed, there are some response elements for the p53 family members in Dicer promoter that may allow its regulation. Moreover, p73 may interact through its binding domain PY with the WW domain of DGRC8, leading to its regulation *(Boominathan and al., 2010).* TRIM32, an ubiquitin ligase, is implicated in Ago1 activation, to promote a specific miRNA process (Let-7, miR-134, miR-214, miR-181). Interestingly, bioinformatic analysis of TRIM32 promoter reveal putative binding site for p73. In addition, the results of microarray show that the expression of TRIM32 is correlated with that of TAp73 *(Rosenbluth and al., 2008)*.

Currently it is difficult to identify specific miRNAs regulated by the p73 isoforms. This is explained by the lack of tools to study them and the lack of specific p73 response elements described, the majority being shared with p53.

V. OBJECTIVES

The main obstacles to the development of more effective treatment for the gastric cancer are the delay in diagnosis, the lack of knowledge of the mechanisms involved in the frequent resistance to anti-cancer drugs and the lack of prognostic molecular markers, allowing the treatment adaptation. Our group studies the molecular mechanisms involved in the progression and aggressiveness of stomach cancer, focusing on the epigenetic modifications, the p53 family members and how gastric cancer cells respond to current chemotherapy such as cisplatin. So my thesis project includes three complementary parts that have a common goal to improve our knowledge on current chemotherapy resistance mechanisms such as cisplatin and to develop new therapies with ruthenium organometallic compounds.

1. Identification of signaling pathways deregulated in GC by cisplatin, a reference drug, and the ruthenium compounds (RDC), a drug being developed, using microarray data.

2. Characterization of certain signaling pathways deregulated in GC in response to cisplatin, as the histone deacetylase (HDAC) and miRNAs, and determined their interaction with the p53 family.

3. Establishment of a combination therapy protocol in GC using a reference therapy (cisplatin, oxaliplatin) and a developing therapy (inhibitor of HDACs).

VI. RESULTS (OBJECTIVE 1)

1. A ruthenium anticancer compound interacts with histones and impacts differently on epigenetic and death pathways compared to cisplatin

A ruthenium anticancer compound interacts with histones and impacts differently on epigenetic and death pathways compared to cisplatin

Licona Cynthia[1#], Spaety Marie-Elodie[1#], Capuozzo Antonella[1,7], Ali Moussa [5], Rita Santamaria[6], Armant Olivier[2], Delalande Francois[5], Van Dorsselaer Alain[5], Sarah Cianferani[5], Spencer John[3], Pfeffer Michel[4], Mellitzer Georg[1], Gaiddon Christian[1]

[1]INSERM 1113, Molecular Signaling of the Cell Stress Response and Pathology, Inserm U1113, Université de Strasbourg, Section Oncologie FMTS, 3 av. Molière, 67200, Strasbourg, France

[2]Karlsruhe Institute of Technology (KIT), Institute of Toxicology and Genetics (ITG), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

[3]Department of Chemistry, School of Life Sciences, University of Sussex, Falmer, Brighton, East Sussex, BN1 9QJ, UK

[4]Institut of Chemistry, UMR7177 CNRS, Université de Strasbourg, Laboratory of metal-induced synthesis, 4 rue Blaise Pascal, 67000, France

[5] Institut Pluridisciplinaire Hubert Curien, Département Sciences Analytiques, CNRS UMR7178, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg Cedex 2, France

[6]Department of Pharmacy, University of Naples Federico II, Naples

Corresponding author: Christian Gaiddon, gaiddon@unistra.fr

[#] Indicates that both authors contributed equally to this work.

Short title: Ruthenium drug transcriptome points to epigenetic

Key words: epigenetic, ruthenium, anticancer, p53, ER stress, transcriptome, cisplatin, HDAC,

Abstract

Ruthenium complexes are considered as potential replacements for platinum compounds in cancer therapy. Their clinical development is handicapped by a lack of consensus on their mode of action. In this study, we identify three histones (H3.1, H2A, H2B) and the histone binding protein as possible targets for an anticancer redox organoruthenium compound (RDC11). Using purified histones we confirmed a direct interaction between RDC11 and histones that impacts on histone complex formation. A comparative study of RDC11 with cisplatin shows differential histone epigenetic modifications that correlate with differential expression of histone deacetylase (HDAC) genes. To characterize the impact of these epigenetic modifications on signaling pathways we performed a transcriptomic study. Clustering analyses showed gene expression signatures specific for cisplatin (42%) and for RDC11 (30%). Signaling pathway analyses pointed to specificities distinguishing RDC11 from cisplatin. For instance, cisplatin triggers preferentially p53 and folate biosynthesis while RDC11 induces reticulum endoplasmic stress and trans-sulfuration pathways. To further understand the role of HDAC in these regulations, we used SAHA and showed that it synergizes with cisplatin cytotoxicity while antagonizing RDC11 activity. This study provides critical information for the characterization of signaling pathways differentiating both compounds. In particular, by the identification of a non-DNA direct target for a ruthenium complex.

Introduction

Transition metal complexes, including those of ruthenium, have been under investigation for several years as scaffolds for generating novel molecules harboring anticancer properties. These metals present interesting properties that provide advantages for designing cytotoxic compounds such as: enabling, otherwise inaccessible to carbon-based chemistry, an octahedral geometry, a wide variety of redox potentials, accessibility of numerous oxidation states (I to IV) and interesting ligand exchange rate allowing covalent interactions with biological macromolecules (1,2). In addition, ruthenium is hypothesized to be less toxic than platinum as it might be eliminated by iron metabolism mechanisms. A multitude of ligands have been used to produce different ruthenium complexes in the redox state (II) or (III). Most of the compounds were generated through complexion with a nitrogen atom within the ligand. Alternatively, organo-ruthenium compounds were generated via a covalent bond with a carbon (C) of the ligands. *In vitro* and *in vivo* biological studies established that several of these ruthenium-based compounds show high cytotoxicity towards a wide range of cancer

cells and reduced side effects (1-12). Gratifyingly, ruthenium-based complexes are not affected by platinum-induced resistance mechanisms. Based on these characteristics, two ruthenium-based complexes, NAMI-A and KP1019, have been tested in phase I and II clinical trials (13,14). However, the lack of success of ruthenium compounds in late stage clinical trials may reside in part in the relative lack of understanding of their exact mode of action and the important chemical determinants involved.

In this respect, the mechanism of action of ruthenium-based complexes remains a matter of debate. Several modes of action have been proposed, which include interaction with DNA and activation of DNA damage pathways (15-19), inhibition of kinases (20) or other enzymatic activities (21,22), including extracellular metallo-proteases (23). This variability may be due to differences in the structure of the ruthenium complexes, which reside in the variation of the nature of the ligands but also the type of bond linking the ligand to the ruthenium atom. In addition, no global approaches have been described so far that would give a more exhaustive and comprehensive understanding of the signaling pathways that are triggered in response to ruthenium-based compounds.

In this study, we have analyzed direct protein targets of RDC11 and changes in gene expressions caused by this complex in comparison to the well-established anticancer metal-based drug cisplatin. RDC11 is an organo-ruthenium compound in which two acetonitriles, one phenanthroline, and one 2-phenylpyridine ligand are linked to the metal. The 2-phenylpyridine is cyclometalated to the ruthenium, i.e. it is bound to Ru via the nitrogen's lone pair and an ortho carbon atom of the phenyl unit. We previously demonstrated that RDC11 is highly cytotoxic (IC$_{50}$ between 1-5µM) on multiple cell lines including cisplatin resistant cells (18,24). Importantly, RDC11 reduces tumor growth in different models, including mouse syngeneic models (melanoma, lung cancer) and human xenografted models (glioma and ovarian cancer), with a reduced toxicity towards healthy tissues compared to cisplatin (24,25). We have also previously shown that RDC11 and related compounds such as RDC34 induce p53-dependent and Endoplasmic Reticulum (ER) stress pathways. However, we also showed that both pathways could not account for all the biological effect of RDC11-related compounds (18,25,26). Finally, structure activity studies have indicated that RDC11 and RDC34's cytotoxicity is at least partly related to their redox potential and the production of reactive oxygen species (24,27).

To understand the mode of action of RDC11 we have used a proteomic approach identifying histones as potential proteins targeted by RDC11 and we have established the impact on the cellular transcriptome to identify novel signaling pathways that could elucidate the biological activity of RDC11. In addition, we have performed this analysis comparatively to cisplatin in

order to characterize specific signatures or similarities between cisplatin and our organoruthenium compound.

Materials and methods

Chemicals

Cisplatin, pifithrin, SAHA and salubrinal were purchased from Sigma. RDC11 was synthesized and purified as previously described (18).

Cell cultures

U87, AGS and HCT116 cells were obtained from ATCC. Cells were manipulated and cultured in DMEM with 10% FCS (Dominique Dutcher^{TM}) and 1% Penicillin + Streptomycin (Sigma) at 37°C with 5% CO_2 atmosphere as previously described (28).

Cell survival assay

2000 cells were seeded per well in 96-well microplates (Falcon Multiwell), 48 h prior to any treatment. Cisplatin and RDC11 were applied for 48 h in fresh medium. MTT assay was performed as previously described by replacing the medium with fresh medium supplemented with 5 mg/L MTT (Sigma) for 1h (29). Cells were lysed in isopropanol with 0.04 N HCl. Measurements were performed at 550 nm.

Quantitative PCR.

Cultured cells were lysed with 1ml of TRIzol (Invitrogen) per 10×10^6 cells and RNA extracted according to manufacturers instructions. RNA samples were ethanol-precipitated twice and 1 µg was used for reverse transcription (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). qPCR was performed using 2 ng/µl cDNA (RNA equivalent) according to manufacturers' instructions (SYBR Green PCR Master Mix, Applied Biosystems) and with 400 µM of each primer (Supplemental data, Table 1). Expression levels were normalized using average of 18S.

Statistical analysis

Statistical analyses were performed using a one-way ANOVA test followed by a Tukey post-test allowing a comparison between all the conditions. * Indicate statistical differences in graphs. Statistical Analyses were performed using Prism (GraphPad software).

Western blotting.

Cells or tissue were lysed with LB (125mM Tris-HCl pH 6.7, NaCl 150mM, NP40 0.5%, 10% Glycerol). Proteins (20µg) were denatured and deposited directly (75µg of proteins) onto a SDS-PAGE gel. Western blotting was performed using antibodies raised against p53 (rabbit anti-p53, FL-393, Santa Cruz, CA), XBP1s (Santa Cruz, CA), EZH2 (Cell Signaling), H3 (Cell Signaling), H3K9 (Abcam) and HDAC4 (Biolegend). Secondary antibodies (anti-rabbit, anti-mouse: Sigma, MA) were incubated at 1:1000. Loading was controlled with actin (rabbit anti- β-actin, Sigma, 1:4000) (30).

Results

The cytotoxic organoruthenium complex RDC11 interacts with histones in cancer cells

We previously showed that although RDC11 interacts with DNA this does not fully explain its anticancer activity (24,25,31). To identify RDC11's putative protein targets we used an affinity chromatography approach in which RDC11 is covalently bound to solid matrix (HypoGel 400-COOH) (Supplementary information S5). As a source of possible protein targets we used cell extracts of gastric cancer AGS cells. AGS cells are more sensible to RDC11 than to cisplatin (Figure 1A, B). Before loading, AGS cell extracts were treated with DNAse to maximize the liberation of DNA bound proteins. After incubation of the AGS cell extract for 1h with the RDC11-matrix, the matrix was washed several times with buffer of increasing salt concentration and then proteins were eluted with a solution of free RDC11. Eluted proteins were identified using mass-spectrometry. The experiment was repeated several times and the results were each time compared to proteins identified using only the naked matrix. Three histones, H3.1, H2A.1B and H2B.1K, and histone binding proteins, RBBP4 and RBBP7, were found repeatedly to bind to the RDC11-matrix (Figure 1C). Despite their small molecular weight, for each histone several peptides were identified by mass-spectrometry. As histones are DNA binding proteins and we previously showed that RDC11 binds to DNA, we decided to confirm that DNA did not mediate the interaction between RDC11 and histone. To do so, we used purified histones and incubated them with increasing amount of RDC11. The mixture was then put to migration in a SDS-page under non-denaturing conditions (Figure 1D). Purified histones can migrate in non-denaturing gels as monomer, dimer and trimers. In the presence of RDC11, histone H3 migrated slower and with a more diffuse pattern suggesting the presence of histones of higher molecular weight due to the binding of RDC11. Changes in histones migration were observed with histone monomers, dimers and trimers.

In cells, histones are the subject of multiple modifications that modulate their association with DNA, DNA compaction and also their translocation into the cytoplasm (32). To assess the impact of RDC11-histone interactions in cells, we treated gastric cancer cells with RDC11 and extracted histones using two different protocols that allow either a preferential extraction of a soluble histone fraction (NP40 extraction buffer) or a full extraction of the histones bound to DNA (sample buffer) (Figure 1E). We observed that already after three hours of treatment with RDC11 at the IC_{50} or the IC_{75} values, the soluble fraction of histone H3 was significantly reduced. All together these results indicated that RDC11 can bind directly to histones and that this binding can impact on histone function in cancer cells.

Differential impact of RDC11 and cisplatin on histone modifications and epigenetic processes

As RDC11 binds to histones and diminishes the more soluble fraction of histone we hypothesized that it might impact on the post-translational modifications of histones. We analyzed by Western-blot the acetylation of histone H3 at lysine 9. We observed that histone H3 acetylation was strongly increased by cisplatin and RDC11 already 6 hours after treatment. However, after 24 hours there was a significant decrease of histone H3 acetylation under the RDC11 conditions while it remained elevated with cisplatin (Figure 2A).

To further understand the impact of RDC11 and cisplatin on epigenetic regulations related to histones, we analyzed the expression of several genes encoding for enzymes involved in histone modifications. RT-qPCR experiments indicated that RDC11 and cisplatin regulated differently the expression of several histone deacetylases (HDAC) or other enzymes such as EZH2 (Figure 2B). In particular the expression of HDAC4 was repressed by cisplatin and not affected by RDC11 while EZH2 was induced. In addition and conversely to HDAC4, HDAC9 was induced by RDC11 and not by cisplatin. At the protein level, RDC11 induced HDAC4 at 6 hours while cisplatin already diminished its protein levels (Figure 2C). Finally, we used a xenografted tumor model to establish that the inhibition of HDAC4 expression was also observed *in vivo* (Figure 2D).

The organoruthenium complex RDC11 presents a distinct transcriptomic signature

The ability of RDC11 to interact directly with histones and its impact on histone post-translational modification through epigenetic regulation suggested that RDC11 might alter a broad range of signaling pathways. Hence to identify without bias RDC11 deregulated signaling pathways we performed a transcriptome analysis. In addition, to identify similarities and specificities in the gene regulations caused by ruthenium-based complexes versus platinum-based complexes, we treated cancer U87 cells at the IC_{50} values for RDC11 (2μM)

and cisplatin (3µM) (Supplementary information S3A). We chose these cells because the IC_{50} of the two drugs are close allowing us to identify gene regulations under similar treatment conditions. We also tested two time points, 6h and 24h, in order to identify early and later events in the gene regulations and their possible temporal and functional relationships. For the identification of regulated genes, we used Affymetrix hugene10stv1 arrays and each condition (Control: Ct; RDC11 6h: R6; RDC11 24h: R24; cisplatin 6h: C6; cisplatin 24h, C24) was performed in triplicate.

Principal component analysis of the normalized data using RMA and probe-level linear models validated that the data are reproducible (Supplementary information Fig. S1A, S2, S3B). To detect differentially expressed genes, we performed the comparative analysis between control and treated groups 6 hours and 24 hours after exposure. 4540 probe sets with fold change ≥ ±1.5 and adjusted p-value ≤ 0.05 were considered as significantly regulated in at least one comparative analysis. To find groups of genes with similar expression pattern across the different conditions and thus potentially involved in the same regulatory pathways, we performed hierarchical clustering analysis on the set of 4540 de-regulated probe-set (Figure 3). The analysis of the 4540 genes revealed that 922 probe-sets are de-regulated by cisplatin and 748 probe-sets by RDC11 6 hours after exposure (Figure 4A, Supplementary information Fig. S1B). After 24 hours, 2910 and 2314 probe-sets are regulated by cisplatin and RDC11 respectively (Figure 4A). 22% to 28% (n=207) of the probe-sets are regulated both by RDC11 and cisplatin 6 hours after treatment. These numbers increase to 38% to 48% (n=1107) by 24 hours (Figure 4A).

Several relevant groups of genes are deregulated. Differences in gene regulation are already detectable 6 hours after exposure. For instance the cluster 10, 4 and 15 are made of genes downregulated at 6 hours of cisplatin treatment compared to control. Overall, the effect on gene regulation is higher after 24 hours both in term of genes regulated and differences in gene expression levels (Figure 3). One group of genes (cluster 6, n=776) is characterized by low/moderate gene expression levels 6 hours after treatment with RDC11 or cisplatin, and high expression levels after 24 hours treatment. Gene set enrichment analysis revealed that this cluster is highly enriched for genes with functions in apoptosis (FDR < 10^{-5}) such as p21, IRF1, NFKBIB, I-kB, ATF-3, PP2A regulatory, Caspase-7, C/EBP zeta, NF-kB, PPP2R5A, suggesting that apoptosis is induced by both chemicals at 24 hours. In addition to this common set of genes, clustering revealed a specific group of genes induced either by RDC11 or cisplatin. For instance the cluster 8 (n=753), which is characterized by high expression levels 24 hours after cisplatin treatment, is enriched for genes in "apoptosis and survival Apoptotic Activin A" signaling (FDR < 10^{-3}) such Activin A, ActRIIA, Bcl-XL, p53, H-Ras, SHIP, c-Fos, while

this pathway is not significantly regulated in by 24h RDC11 treatment (R24). Cluster 7 (n=409) is another example highlighting intrinsic differences in the transcriptional changes induced by RDC11 and cisplatin with a group of genes highly expressed in R24 and with moderate or low expression levels in other conditions. Some effect of the cisplatin is mediated by specific down regulation of a set of 710 genes (cluster 10) enriched for function in cell adhesion via ephrin signaling (FDR < 10^{-5}) such Ephrin-A, Tiam1, FAP-1, Ephrin-A5, Fyn, GRB10, Intersectin, VAV-2, NCK2 (Grb4), FAK1 (Supplementary table T1). Clustering into the different groups was confirmed by soft clustering (Supplementary information Fig. S1C) (33). This, Identifying again a group of genes, soft cluster A, showing a moderate decrease in their expression at 6 hour, which is further downregulated at 24 hours of treatment with cisplatin or RDC11, respectively. Another example for the different effect of cisplatin and RDC11 on a group of genes is soft-cluster B, where cisplatin shows only a very low/moderate effect on their expression, whereas this group of genes is strongly upregulated by RDC11 at 24 hours of treatment. Together, these data suggest that cisplatin and RDC11 share some mechanism activating apoptosis, but overall the mechanism of actions of these two drugs is distinct. Indeed, although the absolute number of genes regulated in common by both chemicals reached a maximum of 48% 24 hours after the treatment (Figure 4A), the levels of regulation as well as the early and late responses are different, making the transcriptional imprint unique for each chemical in the condition tested here.

In order to assess a possible relative generalization of the findings to other cancer cell types and to validate some of the regulations observed using the arrays, we performed RT-qPCR analyses on U87 cells, HCT116 colon cancer cells and AGS gastric cancer cells. For this, several genes were chosen randomly and a representative set is shown in supplementary information figures S3C and S4. Results were compared to the data obtained by the microarray analyses. In each cell line, drugs were applied at their IC_{50} value for 6 or 24 hours. RT-qPCR experiments showed a good correlation with the results of the arrays for U87 or HCT116 cells. However, the correlation was not so strong for AGS cells, indicating that some of the mechanisms might be cell line-specific.

The organoruthenium complex RDC11 modulates pathways distinct from cisplatin

As indicated above, gene set enrichment analyses of different databases identified pathways commonly or differently affected by RDC11 and cisplatin. Figure 4B shows the number of genes identified by gene set enrichment analyses that are upregulated in RDC11 treated cells for the indicated pathway between 6 hours and 24 hours when compared to control conditions. As already seen in the clustering analyses (Figure 3), the overall regulations

intensified with time, such as genes involved in apoptosis (i.e. APAF1, Caspase 3). In correlation with apoptosis-related genes, several genes involved in selected pro-apoptotic pathways, such as p53 target genes, ER stress-related and oxidative stress-related genes are induced over time. Importantly, the expression of several genes of these pathways is already induced at 6 hours. In addition, genes involved in other cellular processes, such as several DNA damage-related genes, a few miRNAs, and multiple genes encoding enzymes involved in epigenetic control are upregulated over time by RDC11.

Interestingly, when we compared the number of genes involved in these pathways and present at 24 hours in RDC11 or cisplatin treated cells, several differences can be identified (Figure 4C). For example, p53-, DNA damage- and apoptosis-related genes are more present in cisplatin-treated cells. Inversely, ER stress- and oxidative stress-related genes are more frequent in RDC11-treated cells. Note that the number of miRNAs and epigenetic-related genes present in RDC11- and cisplatin-treated cells is similar. Strikingly, gene set enrichment analyses revealed that several cellular metabolic processes were very selectively regulated by either RDC11 or cisplatin (Figure 4D). For example, trans-sulfuration and aminoacyl tRNA synthetase are selectively induced by RDC11. In contrast, genes involved in sterol biosynthesis and sucrose metabolism are preferentially regulated in cisplatin-treated cells.

All together the gene set enrichment analyses indicated that RDC11 and cisplatin present each a preference in signaling pathways they regulate. These results clearly demonstrate that cisplatin and the ruthenium-based complex RDC11 have a different mode of action and pinpoint to some potential mechanisms that may account for the cytotoxicity of RDC11.

Cisplatin is a more potent inducer of the p53 pathway than the organoruthenium complex RDC11

To further validate some of the compound specific signatures identified by the gene set enrichment analyses, we performed RT-qPCR to measure the expression of p53 target genes in HCT116. Cells were treated for 6 hours and 24 hours with cisplatin or RDC11 at their IC$_{50}$. *Gdf15*, *fas*, *bak1* and *plk3* were all induced by cisplatin and RDC11 at 24 hours (Fig. 5A, B, C, D). However, the induction by cisplatin was stronger and occurred often already at 6 hours of treatment. We then followed the protein level for p53 under the same experimental conditions. P53 proteins were more abundant in cisplatin-treated cells compared to RDC11-treated cells (Fig. 5E). In addition, increased p53 protein expression was also seen at 6 hours in cisplatin-treated cells. To assess the importance of p53 in RDC11 biological activity, we used pifithrin, which is considered to be a p53 inhibitor (34). Survival of cancer cells was assessed using MTT assays. Pifithrin did not block the biological activity of RDC11 except at doses

above the IC_{75} values (75 % of the maximal effect of RDC11, Fig. 5F). The results indicated that RDC11 is less capable of inducing p53 signaling than cisplatin, but p53 may still play a role in the biological activity of RDC11.

RDC11 is a more potent inducer of the ER pathway than cisplatin

We also focused on the ER stress pathway that represented a more selective signature for RDC11. RT-qPCR showed that several genes, *ditt3*, *atf4*, *chac1*, *dnajb2*, involved in the ER stress pathway were preferentially induced by RDC11 (Fig. 6A, B, C, D). Some of these genes were also induced by cisplatin, but to a significantly lesser extent. To further document the induction of the ER stress pathway we analyzed the production of the spliced form of XBP1 (XBP1s). XBP1s is generated from IRE1 that is itself activated by the ER stress pathway. Figure 6E shows that RDC11 strongly induced the production of XBP1s while cisplatin had no or little effect. To assess whether eif-2α, a component of the ER stress pathway, is essential in the biological activity of RDC11 we used salubrinal, a known inhibitor of eif-2α (35). Cell survival was assessed by a MTT assay and revealed that salubrinal did not significantly alter the cytotoxicity of RDC11 (Fig. 6F). These results showed that although RDC11 stimulates preferentially the expression of components of the ER stress pathway, the activity of eif-2α is not required for RDC11 cytotoxicity.

An HDAC inhibitor antagonizes RDC11 activity but synergizes with cisplatin in gastric cancer cells

The gene set enrichment analyses also showed a strong deregulation of genes involved in epigenetic mechanisms (Figure 4B and C) confirming our previous observations on the effect of cisplatin and RDC11 on HDAC gene expression (Figure 2B, C, D).

To further assess the importance of HDAC regulation in cisplatin and RDC11 biological activity in cancer cells, we used the HDAC inhibitor SAHA. MTT assays were first performed to establish the dose response curve of RDC11, cisplatin and SAHA in AGS cells (Fig. 7A, B and supplementary data). In a second step, combination treatments were performed by combining the two drugs at specific concentrations (i.e. IC_{25}, IC_{50}) accordingly to the isobologram protocol (36). The results were then analyzed using isobologram statistical analyses. This analysis performed with different combinations of HDAC inhibitor and RDC11 showed an antagonistic effect of the HDAC inhibitor on the cytotoxicity of RDC11 (Fig. 7D). In contrast, the HDAC inhibitor synergized with cisplatin cytotoxicity (Fig. 7C). This result showed again a significant difference between the mode of action of RDC11 and cisplatin. In particular, it suggests that HDAC activity might be required for RDC11 cytotoxicity but not for cisplatin.

To understand how SAHA might impact on RDC11 biological activity, we analyzed the p53 and the UPR pathway. As previously observed RDC11 induced p53 and XBP1s protein levels as well as their target genes (Figure 7E, F, G, H). Interestingly, co-treatment with SAHA significantly diminished the impact of RDC11 on both signaling pathways. These series of results indicate that the epigenetic regulation controlled by RDC11 plays a role in RDC11 biological activity and changes the signaling pathways controlled by RDC11.

Discussion

Nowadays, the precise identification of the mode of action of a drug destined for therapeutic purpose is crucial. In oncology, it allows the selection of the group of patients that have the best chance of responding to the treatment in an "a la carte" (personalized) therapeutic approach in which the mode of action of the drug matches the genetic profile of specific tumors. It also provides markers to follow the activity of the drugs during the therapy and point to the direction of a possible combination therapy by combining the compound with other therapeutic molecules. In addition, the identification of the direct target(s) is a clear advantage in developing a rational optimization process aiming at an improvement of the therapeutic activity. The general development of ruthenium-based anticancer compounds toward clinical use has been clearly handicapped by the lack of consensus about their mode of action and the long-lasting misconception that ruthenium-based compounds represent a succedaneum of platinum-based compounds by acting via the same targets. To answer this complex problematic we investigate the possible non-DNA direct targets of a ruthenium based complex (RDC11) and performed an unbiased transcriptomic approach to assess the repercussion of these interactions on cellular signaling pathways key for the control of cell survival. The results of this study provided a comprehensive view on how RDC11 targets histones and illustrate its cellular consequences. In addition, the unbiased and global transcriptomic approach presented in this study provides an unique perspective on the respective characteristics of the organoruthenium compound RDC11 and cisplatin by providing information at two levels: i) a statistical evaluation of the difference and the similarities between the genes regulated by both drugs, ii) a list of novel genes, signaling pathways and metabolic processes regulated specifically by RDC11 that represents a source of information for pathways potentially regulated by other ruthenium-based compounds. Finally, this study illustrates also how such information may provide hints for developing novel combination treatments. In this present case, we focused on histone deacetylase (HDAC) regulation.

An organoruthenium compound interacts with histones

Our study shows that the organoruthenium compound RDC11 interacts with several histones (H3, H2A and H2B) in cells (Figure 1C) and *in vitro* with purified histones (Figure 1D). This finding represents one of the very few examples of a non-DNA target for ruthenium complexes that has been validated using cellular extract. The ability of RDC11 to interact with purified histones suggests strongly that the interaction is direct. In addition, the fact that the interaction is still observed after several washes or in a polyacrylamide gel also indicates that the interaction is relatively stable. It remains to be established more precisely how the compound interacts with histones, such as whether it involves specific interactions and how it affects the proteins at the molecular and structural level. The migration pattern of the RDC11/histone complex shows a tendency of increased trimeric forms, suggesting that the interaction between RDC11 and histone may favor formation of histone complexes (Figure 1D). This is also supported by the fact that using different protein extraction buffers, we observed that treatment of the cells with RDC11 leads to a decrease in the more soluble fraction of histones in the cells (Figure 1E). This set of results supports a precedent finding that a ruthenium complex of piano stool structure called READ-C interacts with histones (37). In addition, our study is the first demonstration of an organoruthenium compounds that interact with histone in cells and it also shows that ruthenium compounds have an impact on the post-translational modifications of histones. These modifications are part of complex and essential epigenetic mechanisms that are essential and complex control of gene expression (32).

An organoruthenium compound impacts on epigenetic regulations.

It is expected that the interaction between a ruthenium compound and histones may alter the complexes in which histones are involved: DNA/histones and histones/epigenetic enzymes modifying histones. Indeed, we observed that in cells treated with RDC11 histones have a different solubility and post-translational modification pattern. For instance, histone H3 levels are lower in the more soluble fraction and its acetylation on lysine 9 is increased (Figure 2). Interestingly, the acetylation pattern is different between RDC11 and cisplatin. It remains to be established whether the modification of solubility and acetylation is directly due to the binding of RDC11 to histone H3. Addressing this specific point represents a complex technical challenge.

Similarly, understanding precisely the relationship between the interaction of RDC11/histones and the transcriptional regulation affecting the epigenetic enzymes will be also challenging. Indeed, if RDC11 alters the expression of HDAC and demethylases (Figure 2B-D, 4B-C), we still need to assess whether these are adaptation/compensation mechanisms of the chromatin to

the structural perturbation due to RDC11 binding, or whether it is a consequence of the modification of chromatin structure leading to different accessibility to transcription factors. Indeed, RDC11 treated cells show a change in the expression of several HDACs and other epigenetic modifiers. These changes are different to those induced by cisplatin. For instance, HDAC4 is strongly repressed by cisplatin whereas RDC11 induced significantly HDAC9. These changes already happen 6 hours after treatment suggesting that they participate into the gene expression regulation observed in cancer cells. The fact that these changes are also observed *in vivo* (Figure 2D) further support their importance for RDC11 and cisplatin's biological and anticancer activity. To elucidate the impact on gene expression of these epigenetic modifications induced by RDC11 and/or cisplatin transcriptomic data were analyzed.

Specificities in RDC11 and cisplatin transcriptomic profiles

Bio-statistical analyses of the microarray data showed significant differences in the number and quality of the genes regulated by RDC11 or cisplatin. Among the 4540 genes that we identified to be regulated, less than a third were common to RDC11 and cisplatin (Figures 3, 4). About 40% were specifically regulated by cisplatin and a third by RDC11. This difference is even more marked at 6 hours post-treatment, as only 1.5% of the regulated genes are common between cisplatin and RDC11-treated cells. The clustering analyses further highlighted the existence of 17 gene clusters that show significant variation of the expression profile over time between RDC11 and cisplatin after treatment (Figures 3, 4). The significant differences in the transcriptome controlled by RDC11 and cisplatin indicate that both compounds should trigger different signaling pathways, likely reflecting distinctive mode of actions.

A more detailed analysis of the signaling pathways or cellular mechanisms that are controlled by the regulated genes confirmed the existence of common and distinctive effects between RDC11 and cisplatin. Apoptosis, DNA damage, p53, epigenetic, miRNA, ER stress and oxidative stress were amongst the mechanisms that were regulated by both drugs (Figures 4, 5, 6). However, the number and the intensity of activation of the genes included in those pathways were often significantly different between RDC11 and cisplatin. For example, cisplatin induces a high number of genes involved in DNA damage, p53, and apoptosis, while RDC11 favors oxidative- and ER stress-related genes. The differences between both drugs are even more accentuated on specific metabolic pathways (Figure 4D). For example, RDC11 specifically regulates ribosome biogenesis, while cisplatin favors sucrose metabolism.

Respective contribution of the p53 and ER stress pathway in RDC11 activity

Some of these specificities were previously documented by our group, such as the preferential activation of the p53 pathway by cisplatin or the ER stress pathway by RDC11 in glioblastoma cells (24,25,31). Here, we further extended these observations by showing that these specificities are common to cancer cells of different origin, such as colon cancer cells (HCT116) or gastric cancer cells (AGS). In addition, we extended the observation to an additional pool of genes and to the protein level for a marker of the ER stress pathway (Figures 5, 6, 7). All together, these novel observations validate the preferential activation of the ER stress pathway by RDC11 in different cancer cell lines. With the endoplasmic reticulum being also the place of protein translation, it is interesting to note that RDC11 triggered a ribosomal biogenesis response along with the ER stress pathway (Figure 5).

Interestingly, the preferential activation of the p53 pathway by cisplatin correlates with a preferential activation of DNA damage response. Hence, compared to RDC11, cisplatin appears to induce a coherent DNA-damage/p53 pathway response, which is also consistent with the preferential binding of cisplatin to DNA as compared to RDC11 (24,25,31).

The exact contribution of the p53 pathway and the ER stress pathways in RDC11 activity have already been investigated previously in glioblastoma cells. Using a dominant inhibitor and p53-/- cell lines, we previously showed that p53 is not absolutely necessary for RDC11 cytotoxicity (24,25). We confirmed here these results by showing that a known inhibitor of p53 (pifithrin) does not drastically alter the activity of RDC11. We also inhibited eIE2⍟, a component of the ER stress pathway, with salubrinal, which did not alter RDC11 cytotoxicity. This result apparently contradicts previous data showing that the ER stress transcription factor CHOP is partly necessary for RDC11 full cytotoxicity (25). This apparent contradiction highlights the complexity of the ER stress pathway and might be explained by the fact that the activation of eIE2α by PERK represents only a part of the mechanisms triggered by the ER stress pathway (38). For example, CHOP can be induced by other mechanisms related to the ER stress pathway, such as ATF6.

Respective role of epigenetic regulators in RDC11 and cisplatin cytotoxicity

Amongst the mechanisms that were regulated differently by RDC11 and cisplatin, several genes encoding for histones and histone-modifying enzymes were included. These genes and in particular HDACs, are playing a critical role in various biological process and represent interesting therapeutic targets (39). Our study showed that whereas EZH2 was induced by both RDC11 and cisplatin, HDAC4 was preferentially repressed by cisplatin. This repression of

111

HDAC4 by cisplatin was also observed *in vivo* in fragments of human colon cancer xenografted in nude mice (Figure 2D). In addition, we observed that these gene regulations impact on the acetylation of histone H3 at lysine 9 in a time- and drug-dependent manner. In particular, 6 hours after treatment, RDC11 represses H3 acetylation while cisplatin already induced it. Such differential drug response correlates with a profound difference in the impact of HDAC inhibitors on RDC11 and cisplatin biological activity. Indeed, a known HDAC inhibitor, SAHA, had a synergistic anticancer activity with cisplatin in AGS human gastric cancer cells as previously observed by others with cancer cell lines of a different origin (40). In contrast, we demonstrate here that an HDAC inhibitor (SAHA) inhibits RDC11 biological activity as revealed by the antagonistic effect observed in the isobologram analysis. This result suggests that, in contrast to cisplatin, RDC11 biological activity might require the activity of some HDACs by modifying either the histones or some of their other targets. From a more therapeutic point of view, this study indicates that RDC11 is not a good candidate to be used in combinatory therapy with general HDAC inhibitors for it might depend on the expression and/or activity of HDACs in the tumor. It also confirms the potential advantage of combining HDAC inhibitors with platinum-based therapies in gastric cancers.

From a more mechanistic point of view, we also show that the combination of RDC11 with SAHA impact on the signaling pathways regulated by RDC11. The induction of p53 and Noxa is almost annihilated by SAHA when combined with RDC11. Similarly, SAHA reduced XBP1s signaling (Figure 7). These changes in signaling may account for the differential biological activity of RDC11 in presence of SAHA.

Overall this study demonstrates that RDC11 and cisplatin have different modes of action and trigger the regulation of different signaling pathways. If both lead to regulation of pro-apoptotic effectors and cell cycle inhibitors, the intermediate mechanisms are quite different. These differences have profound consequences as it will define how we might use RDC11 on tumors with specific signatures (i.e. p53 mutated or not) or with specific other therapeutic molecules. It remains for us to establish how these findings can be generalized to other ruthenium-based compounds or even other metal-based compounds. Our previous studies, as well as those of others showing that ruthenium- or osmium-based compounds with different ligands trigger similar regulation of p53 and ER-stress pathways suggest that the findings of this study might point out potential signaling pathways targeted by other metal-based compounds (24) (19,20,41,42).

Significance

Ruthenium compounds have been developed as a possible replacement for platinum compounds, but only few have reached early phase clinical trial. Their clinical development is handicapped by lack of consensus on their mode of action that is often considered being similar to cisplatin. In this study we identify histones as direct targets for an organoruthenium compound (RDC11) and we performed an unbiased comparative study on gene expression triggered by cisplatin and RDC11. Gene ontology and signaling pathway analyses pointed to major specificities distinguishing RDC11 from cisplatin, including expression change in epigenetic enzymes. This study represents the first approach that summarizes the significant differences in the mode of action of organoruthenium compounds compared to cisplatin.

References

1.   Bruijnincx, P.C. and Sadler, P.J. (2008) New trends for metal complexes with anticancer activity. *Curr Opin Chem Biol*, **12**, 197-206.
2.   Jakupec, M.A., Galanski, M., Arion, V.B., Hartinger, C.G. and Keppler, B.K. (2008) Antitumour metal compounds: more than theme and variations. *Dalton Trans*, 183-194.
3.   Markman, M. (2003) Toxicities of the platinum antineoplastic agents. *Expert Opin Drug Saf*, **2**, 597-607.
4.   Kelland, L. (2007) The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer*, **7**, 573-584.
5.   Allardyce, C.S. and Dyson, P.J. (2001) Ruthenium in medicine: current clinical uses and future prospects. *Platinum Metals Rev.*, **45**, 62.
6.   Dyson, P.J. and Sava, G. (2006) Metal-based antitumour drugs in the post genomic era. *Dalton Trans*, 1929-1933.
7.   Giraldi, T., Sava, G., Bertoli, G., Mestroni, G. and Zassinovich, G. (1977) Antitumor action of two rhodium and ruthenium complexes in comparison with cis-diamminedichloroplatinum(II). *Cancer Res*, **37**, 2662-2666.
8.   Keppler, B.K., Balzer, W. and Seifried, V. (1987) Synthesis and antitumor activity of triazolium-bis(triazole)-tetrachlororuthenate (III) and bistriazolium-triazolepentachlororuthenate (III). Two representatives of a new class of inorganic antitumor agents. *Arzneimittelforschung*, **37**, 770-771.
9.   Sava, G., Pacor, S., Zorzet, S., Alessio, E. and Mestroni, G. (1989) Antitumour properties of dimethylsulphoxide ruthenium (II) complexes in the Lewis lung carcinoma system. *Pharmacol Res*, **21**, 617-628.

10. Fruhauf, S. and Zeller, W.J. (1991) New platinum, titanium, and ruthenium complexes with different patterns of DNA damage in rat ovarian tumor cells. *Cancer Res*, **51**, 2943-2948.

11. Morris, R.E., Aird, R.E., Murdoch Pdel, S., Chen, H., Cummings, J., Hughes, N.D., Parsons, S., Parkin, A., Boyd, G., Jodrell, D.I. *et al*. (2001) Inhibition of cancer cell growth by ruthenium(II) arene complexes. *Journal of medicinal chemistry*, **44**, 3616-3621.

12. Scolaro, C., Bergamo, A., Brescacin, L., Delfino, R., Cocchietto, M., Laurenczy, G., Geldbach, T.J., Sava, G. and Dyson, P.J. (2005) In vitro and in vivo evaluation of ruthenium(II)-arene PTA complexes. *Journal of medicinal chemistry*, **48**, 4161-4171.

13. Hartinger, C.G., Zorbas-Seifried, S., Jakupec, M.A., Kynast, B., Zorbas, H. and Keppler, B.K. (2006) From bench to bedside--preclinical and early clinical development of the anticancer agent indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14A). *J Inorg Biochem*, **100**, 891-904.

14. Rademaker-Lakhai, J.M., van den Bongard, D., Pluim, D., Beijnen, J.H. and Schellens, J.H. (2004) A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. *Clin Cancer Res*, **10**, 3717-3727.

15. Mei, H.Y. and Barton, J.K. (1988) Tris(tetramethylphenanthroline)ruthenium(II): a chiral probe that cleaves A-DNA conformations. *Proc Natl Acad Sci U S A*, **85**, 1339-1343.

16. Brabec, V. (2002) DNA modifications by antitumor platinum and ruthenium compounds: their recognition and repair. *Prog Nucleic Acid Res Mol Biol*, **71**, 1-68.

17. Zeglis, B.M., Pierre, V.C. and Barton, J.K. (2007) Metallo-intercalators and metallo-insertors. *Chem Commun (Camb)*, 4565-4579.

18. Gaiddon, C., Jeannequin, P., Bischoff, P., Pfeffer, M., Sirlin, C. and Loeffler, J.P. (2005) Ruthenium (II)-derived organometallic compounds induce cytostatic and cytotoxic effects on mammalian cancer cell lines through p53-dependent and p53-independent mechanisms. *J Pharmacol Exp Ther*, **315**, 1403-1411.

19. Hayward, R.L., Schornagel, Q.C., Tente, R., Macpherson, J.S., Aird, R.E., Guichard, S., Habtemariam, A., Sadler, P. and Jodrell, D.I. (2005) Investigation of the role of Bax, p21/Waf1 and p53 as determinants of cellular responses in HCT116 colorectal cancer cells exposed to the novel cytotoxic ruthenium(II) organometallic agent, RM175. *Cancer Chemother Pharmacol*, **55**, 577-583.

20. Smalley, K.S., Contractor, R., Haass, N.K., Kulp, A.N., Atilla-Gokcumen, G.E., Williams, D.S., Bregman, H., Flaherty, K.T., Soengas, M.S., Meggers, E. *et al*. (2007) An organometallic protein kinase inhibitor pharmacologically activates p53 and induces apoptosis in human melanoma cells. *Cancer Res*, **67**, 209-217.

21. Ang, W.H., De Luca, A., Chapuis-Bernasconi, C., Juillerat-Jeanneret, L., Lo Bello, M. and Dyson, P.J. (2007) Organometallic Ruthenium Inhibitors of Glutathione-S-Transferase P1-1 as Anticancer Drugs. *ChemMedChem*.

22. Dougan, S.J., Habtemariam, A., McHale, S.E., Parsons, S. and Sadler, P.J. (2008) Catalytic organometallic anticancer complexes. *Proc Natl Acad Sci U S A*, **105**, 11628-11633.

23. Gava, B., Zorzet, S., Spessotto, P., Cocchietto, M. and Sava, G. (2006) Inhibition of B16 melanoma metastases with the ruthenium complex imidazolium trans-imidazoledimethylsulfoxide-tetrachlororuthenate and down-regulation of tumor cell invasion. *J Pharmacol Exp Ther*, **317**, 284-291.

24. Vidimar, V., Meng, X., Klajner, M., Licona, C., Fetzer, L., Harlepp, S., Hebraud, P., Sidhoum, M., Sirlin, C., Loeffler, J.P. *et al*. (2012) Induction of caspase 8 and reactive oxygen species by ruthenium-derived anticancer compounds with improved water solubility and cytotoxicity. *Biochem Pharmacol*, **84**, 1428-1436.

25. Meng, X., Leyva, M.L., Jenny, M., Gross, I., Benosman, S., Fricker, B., Harlepp, S., Hebraud, P., Boos, A., Wlosik, P. *et al.* (2009) A Ruthenium-Containing Organometallic Compound Reduces Tumor Growth through Induction of the Endoplasmic Reticulum Stress Gene CHOP. *Cancer Res*.

26. Klajner, M., Licona, C., Fetzer, L., Hebraud, P., Mellitzer, G., Pfeffer, M., Harlepp, S. and Gaiddon, C. (2014) Subcellular localization and transport kinetics of ruthenium organometallic anticancer compounds in living cells: a dose-dependent role for amino Acid and iron transporters. *Inorganic chemistry*, **53**, 5150-5158.

27. Fetzer, L., Boff, B., Ali, M., Xiangjun, M., Collin, J.P., Sirlin, C., Gaiddon, C. and Pfeffer, M. (2011) Library of second-generation cycloruthenated compounds and evaluation of their biological properties as potential anticancer drugs: Passing the nanomolar barrier. *Dalton Trans*, **40**, 8869-8878.

28. Sohm, F., Gaiddon, C., Antoine, M., Boutillier, A.L. and Loeffler, J.P. (1999) The retinoblastoma susceptibility gene product/Sp1 signalling pathway is modulated by Ca2+/calmodulin kinases II and IV activity. *Oncogene*, **18**, 2762-2769.

29. Gaiddon, C., de Tapia, M. and Loeffler, J.P. (1999) The tissue-specific transcription factor Pit-1/GHF-1 binds to the c-fos serum response element and activates c-fos transcription. *Mol Endocrinol*, **13**, 742-751.

30. Antoine, M., Gaiddon, C. and Loeffler, J.P. (1996) Ca2+/calmodulin kinase types II and IV regulate c-fos transcription in the AtT20 corticotroph cell line. *Mol Cell Endocrinol*, **120**, 1-8.

31. Klajner, M., Hebraud, P., Sirlin, C., Gaiddon, C. and Harlepp, S. (2010) DNA binding to an anticancer organo-ruthenium complex. *J Phys Chem B*, **114**, 14041-14047.

32. Shukla, S. and Meeran, S.M. (2014) Epigenetics of cancer stem cells: Pathways and therapeutics. *Biochim Biophys Acta*, **1840**, 3494-3502.

33. Futschik, M.E. and Carlisle, B. (2005) Noise-robust soft clustering of gene expression time-course data. *Journal of bioinformatics and computational biology*, **3**, 965-988.

34. Gudkov, A.V. and Komarova, E.A. (2005) Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun*, **331**, 726-736.

35. Wiseman, R.L. and Balch, W.E. (2005) A new pharmacology--drugging stressed folding pathways. *Trends in molecular medicine*, **11**, 347-350.

36. Tallarida, R.J. (2006) An overview of drug combination analysis with isobolograms. *J Pharmacol Exp Ther*, **319**, 1-7.

37. Adhireksan, Z., Davey, G.E., Campomanes, P., Groessl, M., Clavel, C.M., Yu, H., Nazarov, A.A., Yeo, C.H., Ang, W.H., Droge, P. *et al.* (2014) Ligand substitutions between ruthenium-cymene compounds can control protein versus DNA targeting and anticancer activity. *Nature communications*, **5**, 3462.

38. Nagelkerke, A., Bussink, J., Sweep, F.C. and Span, P.N. (2014) The unfolded protein response as a target for cancer therapy. *Biochim Biophys Acta*, **1846**, 277-284.

39. Abend, A. and Kehat, I. (2015) Histone deacetylases as therapeutic targets - From cancer to cardiac disease. *Pharmacol Ther*, **147C**, 55-62.

40. Diyabalanage, H.V., Granda, M.L. and Hooker, J.M. (2013) Combination therapy: histone deacetylase inhibitors and platinum-based chemotherapeutics for cancer. *Cancer Lett*, **329**, 1-8.

41. Chatterjee, S., Kundu, S., Bhattacharyya, A., Hartinger, C.G. and Dyson, P.J. (2008) The ruthenium(II)-arene compound RAPTA-C induces apoptosis in EAC cells through mitochondrial and p53-JNK pathways. *J Biol Inorg Chem*, **13**, 1149-1155.

42. Suntharalingam, K., Johnstone, T.C., Bruno, P.M., Lin, W., Hemann, M.T. and Lippard, S.J. (2013) Bidentate ligands on osmium(VI) nitrido complexes control intracellular targeting and cell death pathways. *J Am Chem Soc*, **135**, 14060-14063.

Figure legends

Figure 1: A. Schematic representation of cisplatin and RDC11. B. Survival curve of cancer cells AGS treated with cisplatin or RDC11. Cells were treated for 48 hours in 96-well plates and their survival was evaluated by MTT assay (n=8). C. Table indicating the number of peptides of histones and histone related proteins present in the RDC11 affinity chromatography and mass spectrometry analysis of cell extracts. Repeats are the number of experiment with presence of the peptides out of three experiments done. D. Migration of histone H3-RDC11 complex on non-denaturing SDS-Page. 100ng of histone H3 was incubated with increased concentrations of RDC11 (R 0.05 to 0.25μM) for 1 hour. M is the molecular marker. Image is a silver stained gel of the complex showing the monomeric, dimeric and trimeric forms. E. Proteins were extracted with the indicated buffer (NP40 or sample buffer) from AGS cells treated with RDC11 at the IC50 and the IC75 for 6 hours. Western blot analysis revealed histone H3 and actin protein level.

Figure 2: A. Proteins were extracted from AGS treated with RDC11 (R6, R24) or cisplatin (C6, C24) for the indicated time (6h, R6 and C6; 24h, R24 and C24) using sample buffer. Western blot analysis revealed histone H3 acetyl lysine 7 (H3AK7), histone H3 (H3) and actin expression. Quantifications of H3AK7/H3 are indicated bellow as measured by Pixi imager. B Curves are fold induction versus the control (Ct) for selected histone-modifying enzymes in RDC11 and cisplatin conditions. mRNA levels were assayed in AGS gastric cancer cells by RT-qPCR. Curves are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. C. Proteins were extracted from AGS treated with RDC11 (R6, R24) or cisplatin (C6, C24) for the indicated time. Western blot analysis revealed EZH2, HDAC4, and actin expression. D. mRNA levels of *hdac4* were assayed by RT-qPCR in fragments of human colon cancer xenografted in nude mice and after the treatment with cisplatin or RDC11. Graphs are means of fold induction versus the control (Ct) with SD (n=5). *: p < 0.01.

Figure 3: Hierarchical clustering. Clustering of expression of 4540 probe-set significantly regulated in at least one treated group compared to control. Rows represent gene expression and columns biological samples. Gene expression levels are represented as scaled expression values (row Z-score from -3 to +3). Blue: low expression, Red: high expression, white: moderate expression. 17 different clusters are detected, indicated by their number and different color next the probe-set tree. C6: Cisplatin exposure for 6hrs; C24: Cisplatin exposure for 24hrs; R6: Ruthenium exposure for 6hrs; R24: Ruthenium exposure for 24hrs.

Figure 4: Signaling pathways and mechanisms regulated by cisplatin or RDC11. A. Venn diagram. Significantly mis-regulated probe sets between control and exposed conditions are compared to highlight genes shared or specific to each group. C6: Cisplatin exposure for 6hrs; C24: Cisplatin exposure for 24hrs; R6: Ruthenium exposure for 6hrs; R24: Ruthenium exposure for 24hrs. B, C, D. Graphs represents number of genes in the indicated pathways that are regulated by RDC11 at 6 hours or 24 hours (B) or by RDC11 and cisplatin at 24 hours (C, D). Microarray data were analyzed using AltAnalysis and R bioinformatics tools to identify in KEGG, Gene Ontology, miRNA, transcription factors databanks, the signaling pathways and mechanisms corresponding to the mis-regulated genes.

Figure 5: mRNA levels of *gdf15* (A), *fas* (B), *plk3* (C) and *bak1* (D) were assayed in cancer cells by RT-qPCR. Curves are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. E. Proteins were extracted from cells treated with RDC11 (R6, R24) or cisplatin (C6, C24) for the indicated time. Western blot analysis revealed p53 and actin expression. F. Survival curve of AGS cells treated with RDC11 and **pifithrin-α** (10μM). Cells were treated for 48 hours in 96-well plates and their survival was evaluated by MTT assay (n=8).

Figure 6: mRNA levels of *ddit3* (A), *atf4* (B), *chac1* (C) and *dnajb2* (D) were assayed in cancer cells by RT-qPCR. Curves are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. E. Proteins were extracted from HCT116 treated with RDC11 (R6, R24) or cisplatin (C6, C24) for the indicated time. Western blot analysis revealed XBP1s and actin expression. F. Survival curve of AGS cells treated with RDC11 and salubrinal (10μM). Cells were treated for 48 hours in 96-well plates and their survival was evaluated by MTT assay (n=8). Bars are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01.

Figure 7: Survival curve of AGS cells treated with SAHA (A) and RDC11 (B). Cells were treated for 48 hours in 96-well plates and their survival was evaluated by MTT assay (n=8). Bars are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. Isobologram analyses and combinatory index representations of combinatory treatments between cisplatin with SAHA (C) and RDC11 with SAHA (D). Doses at IC_{75}, IC_{60}, IC_{50}, IC_{30}, IC_{25} were combined and the results were analyzed with the algorithm of Chu and al. using the CompuSyn software. E, G. Proteins were extracted from cancer cells treated with RDC11 (R24) or SAHA (S24) or RDC11 and SAHA (R=S) after 24 hours of treatment. Western blot analysis revealed p53 (E) and XBP1s (G) and actin expression. Quantifications are indicated bellow as measured by Pixi imager F. H. mRNA levels of *p53* (A), *noxa* (B), *ddit3* (C) and *chac1* (D) were assayed in cancer

cells by RT-qPCR. Graphs are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. E. Proteins were extracted from cells treated with RDC11 (R24) or SAHA (S24) or RDC11 and SAHA (R+S) for the 24 hours.
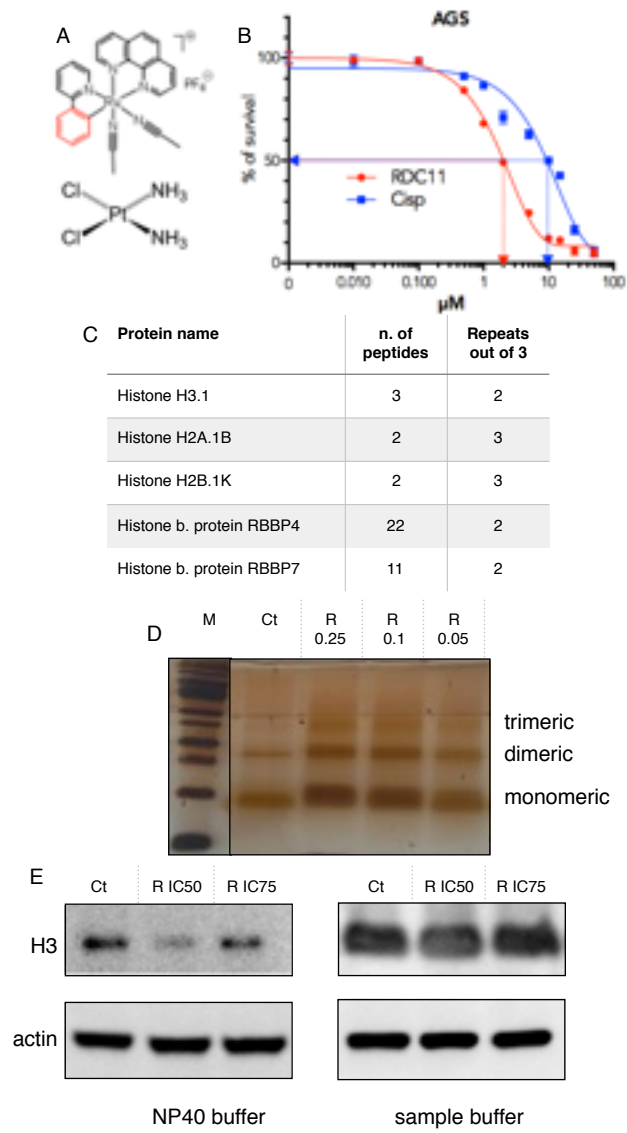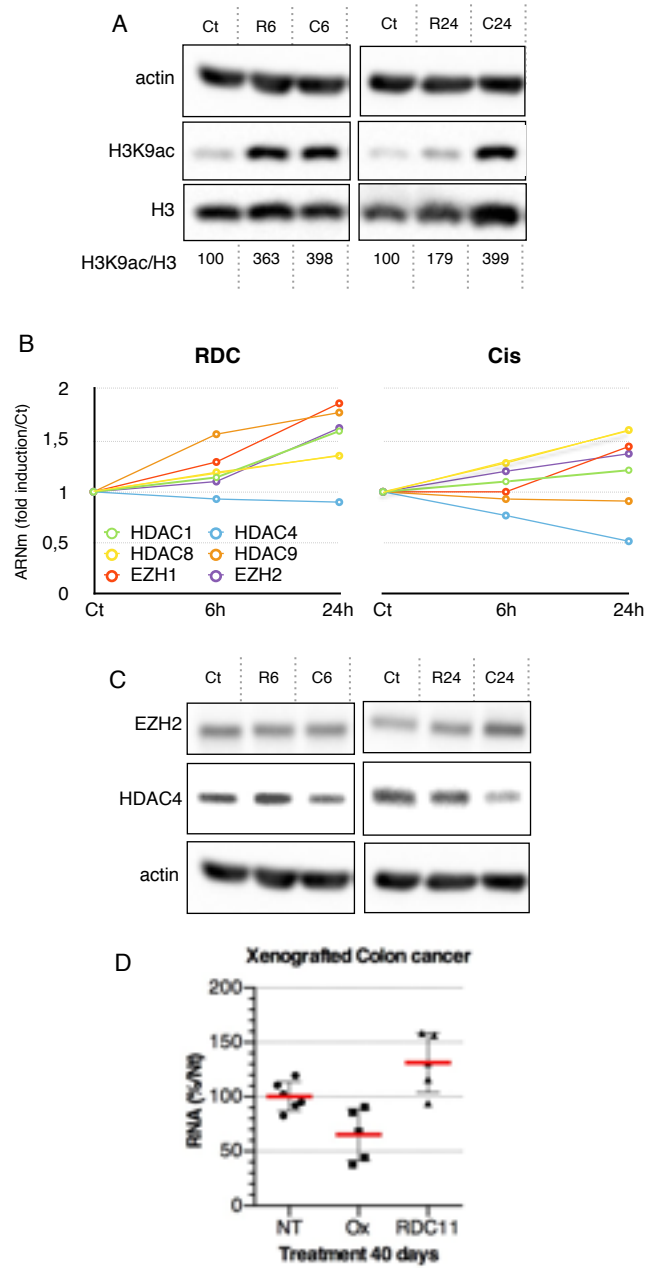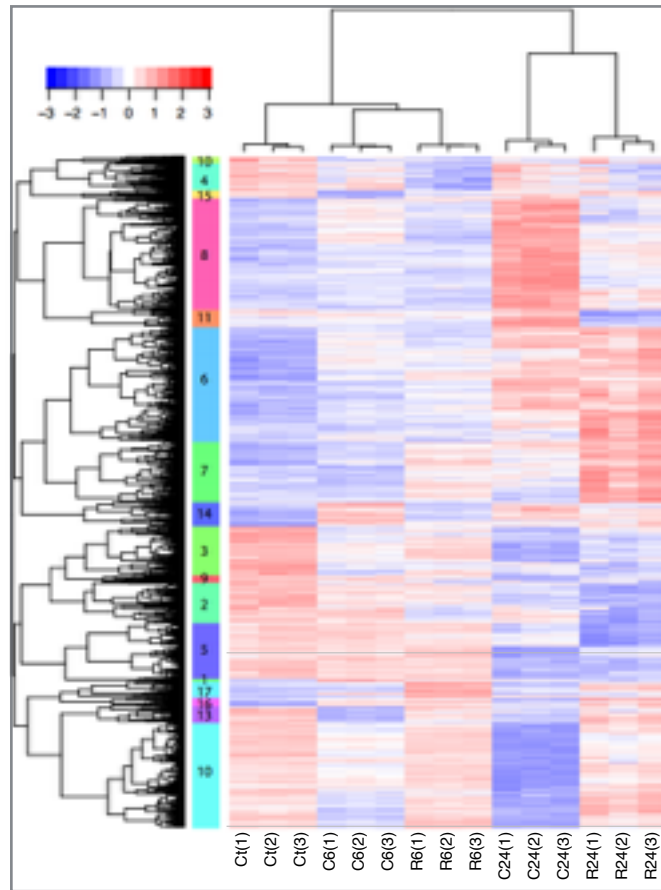
Figure 1

Figure 2

Figure 3

Figure 4
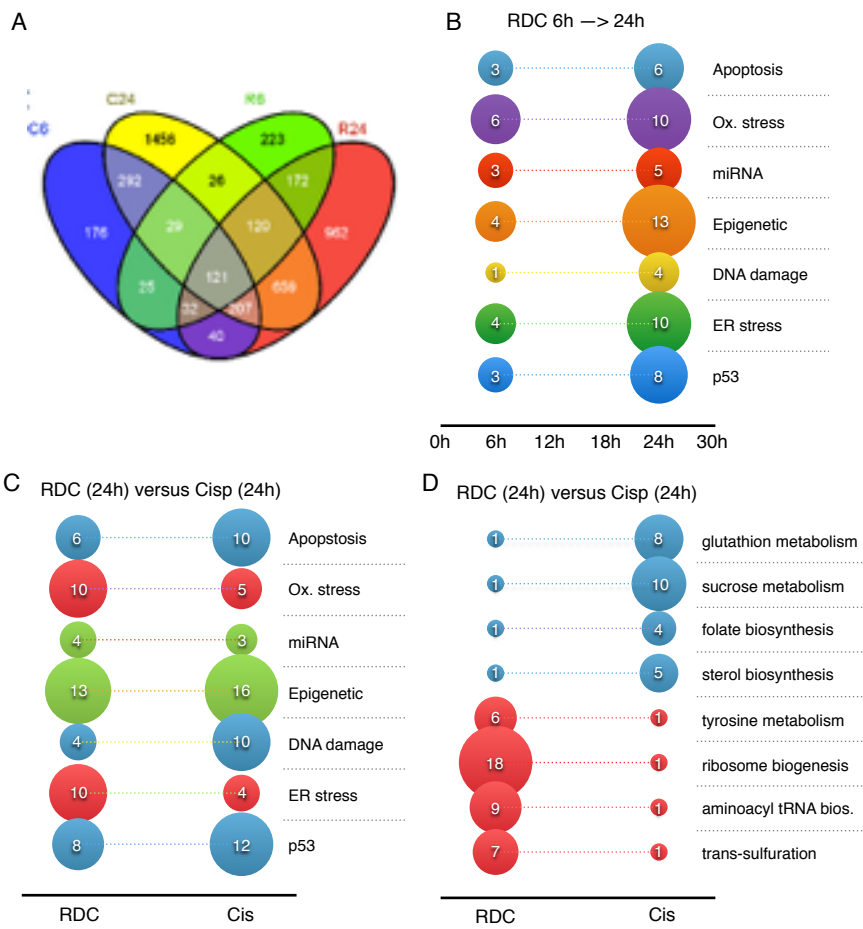
Figure 5
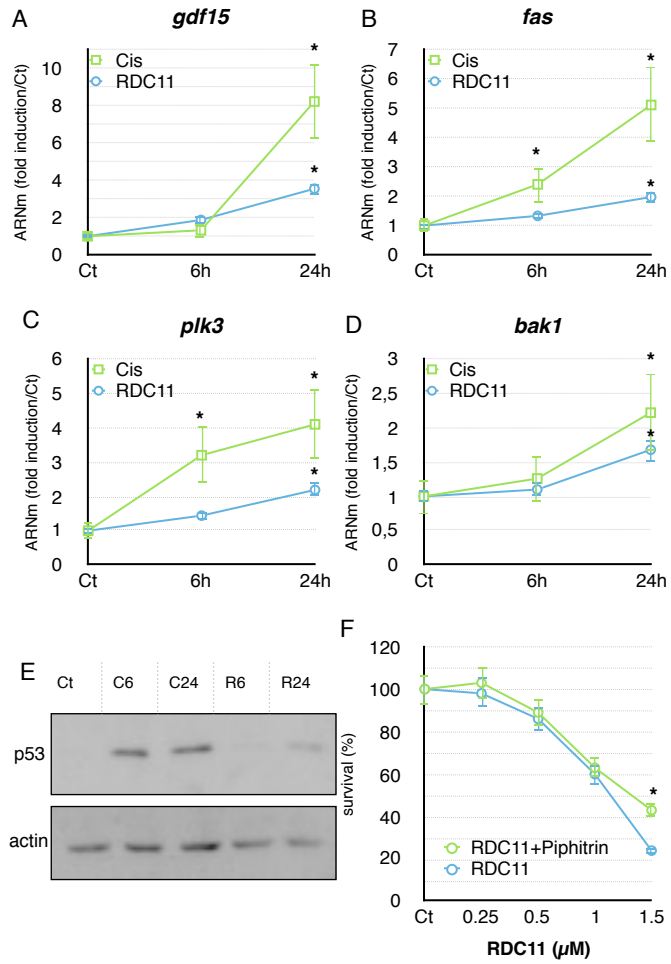
Figure 6
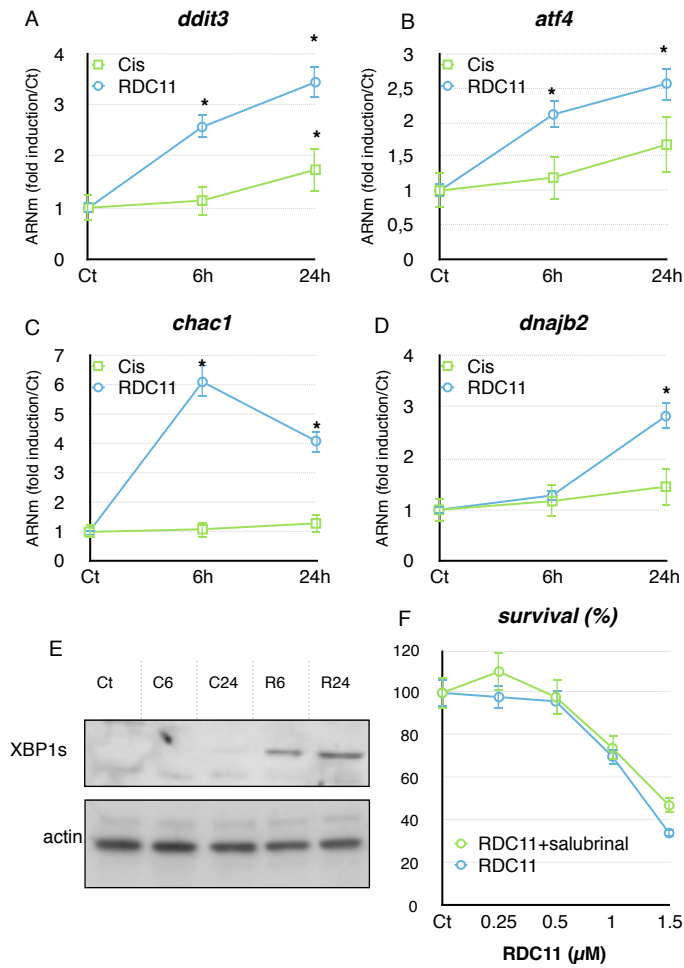
Figure 7
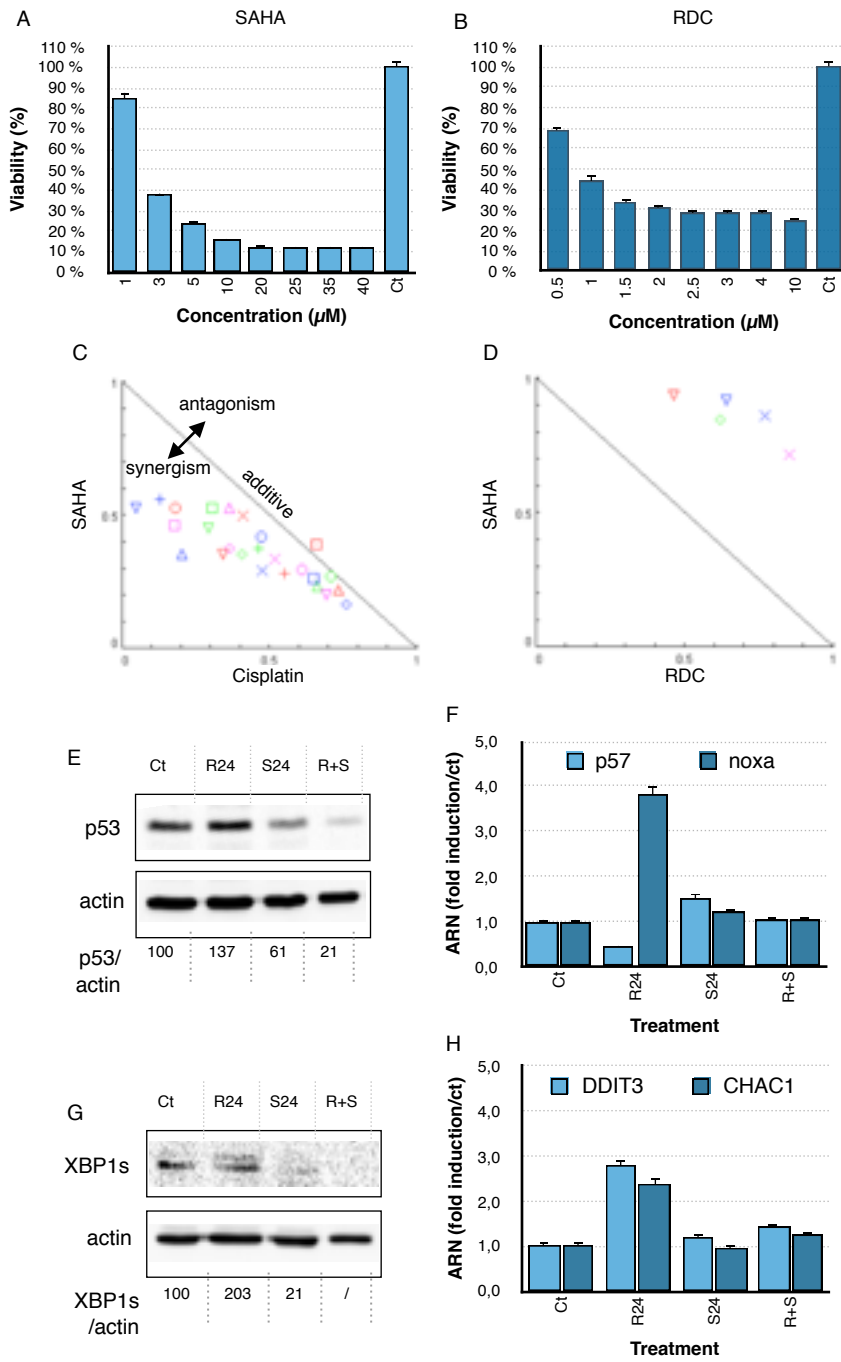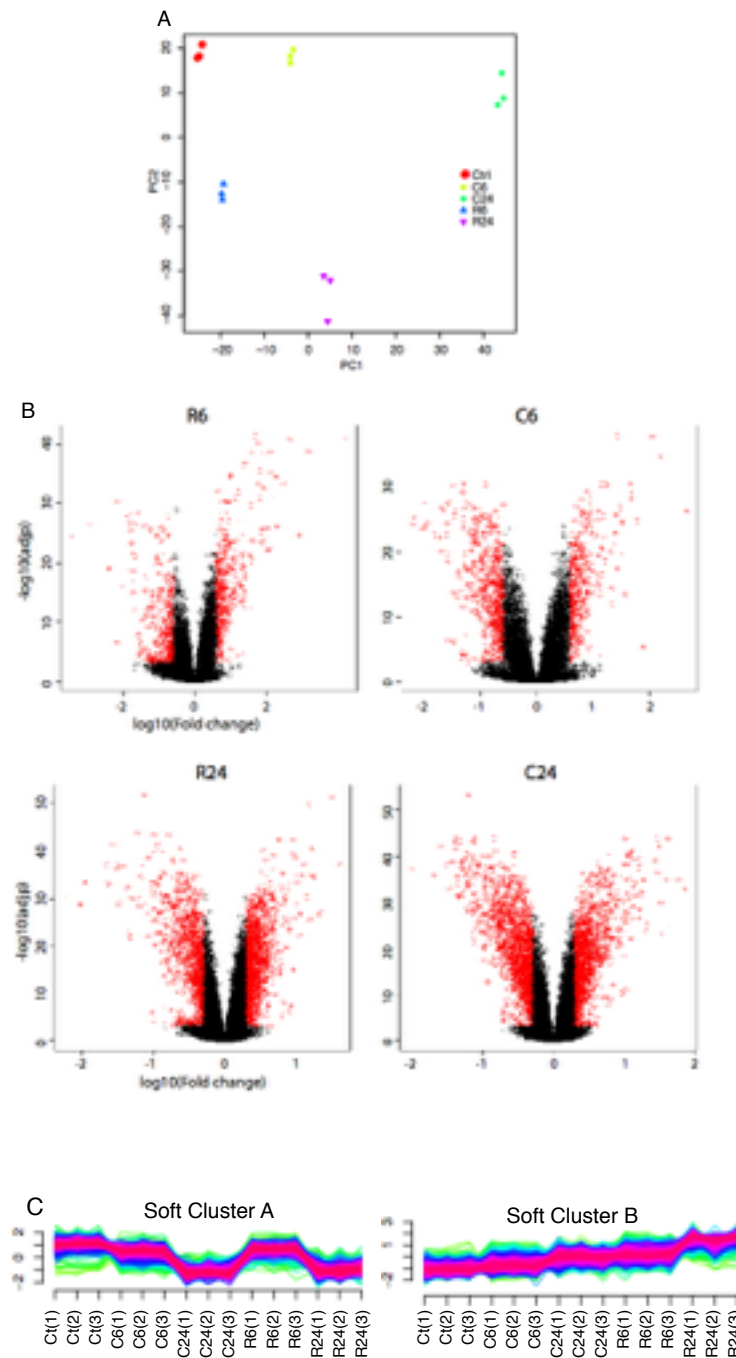
A. Principal Component Analysis. Plotting of two principal components from RMA normalized data using probe probe-level linear models. Biological replicates for the 5 different conditions cluster together and no outliers are detected within the dataset.

B. B. Vulcano plots. Plotting of the log10 of the fold change (x-axis) in function of the -log10 of the adjusted *p* values (y-axis). Unexposed cells are used as reference. Significant differential expression in microarray data are shown in red for the four comparative analysis using the threshold: |FC| ≥ 1.5 and adjusted p-value ≤ 0.05.

C. Soft clustering of the normalized microarray data. The set of 4540 mis-regulated genes was subjected to soft clustering to find gene families sharing similar expression patterns. Genes belonging to the same cluster, ie. with high membership, are colored in red. Normalized and scaled expression levels are indicated on y-axis. Samples names are indicated below each graph.

CGN_C1_HuGene.CEL

CGN_C2_HuGene.CEL

CGN_C3_HuGene.CEL

CGN_C6_1_HuGene.CEL

CGN_C6_2_HuGene.CEL

CGN_C6_3_HuGene.CEL

CGN_C24_1_HuGene.CEL

CGN_C24_2_HuGene.CEL

CGN_C24_3_HuGene.CEL

CGN_R6_1_HuGene.CEL

CGN_R6_2_HuGene.CEL

CGN_R6_3_HuGene.CEL

CGN_R24_1_HuGene.CEL

CGN_R24_2_HuGene.CEL

CGN_R24_3_HuGene.CEL
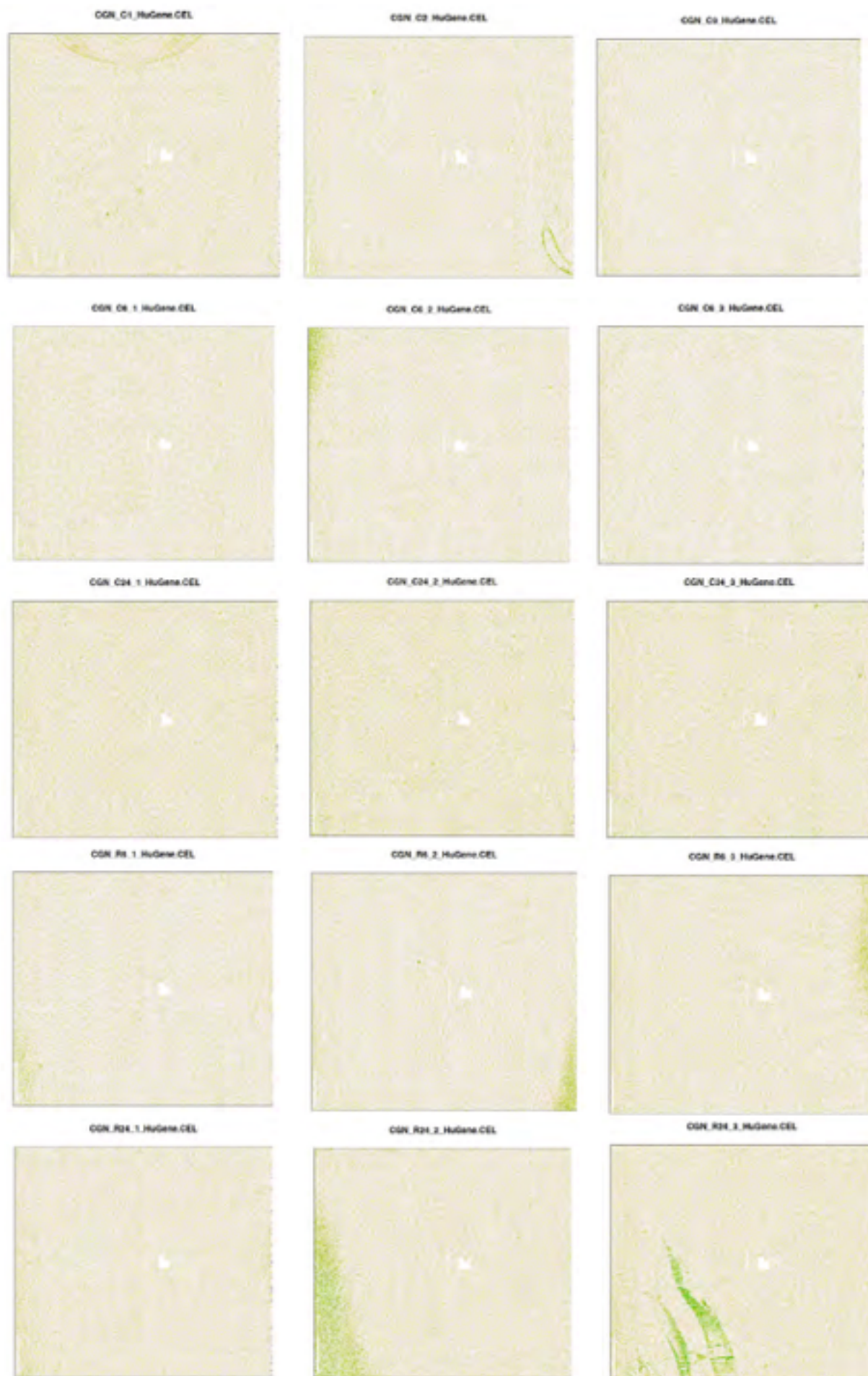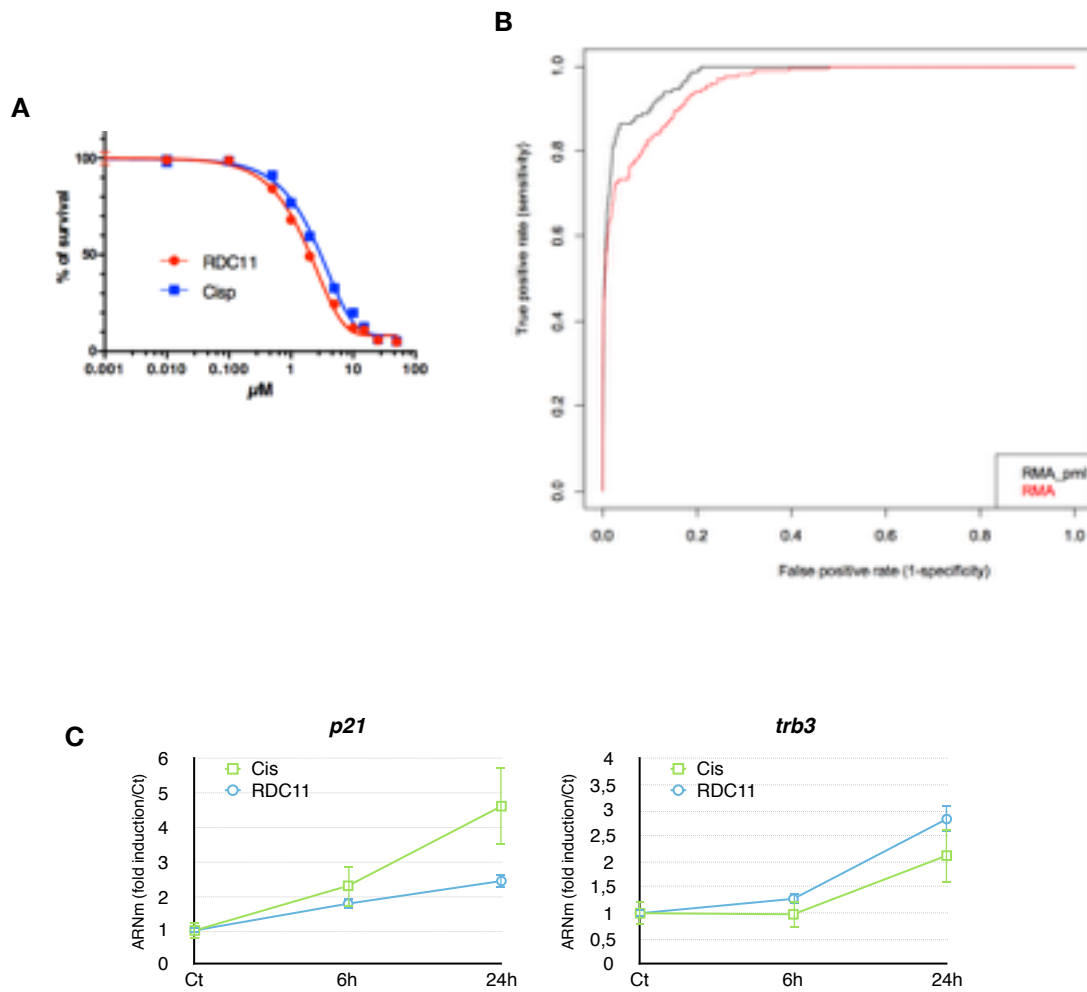
A. MTT of U87 cells treated with RDC11 and Cisplatin for 48 hours. **B**. Graph representing microarray statistical analysis. **C**. mRNA levels of *genes found disregulated in the microarraus: p21* and *trb3* were assayed by RT-qPCR. Curves are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01.
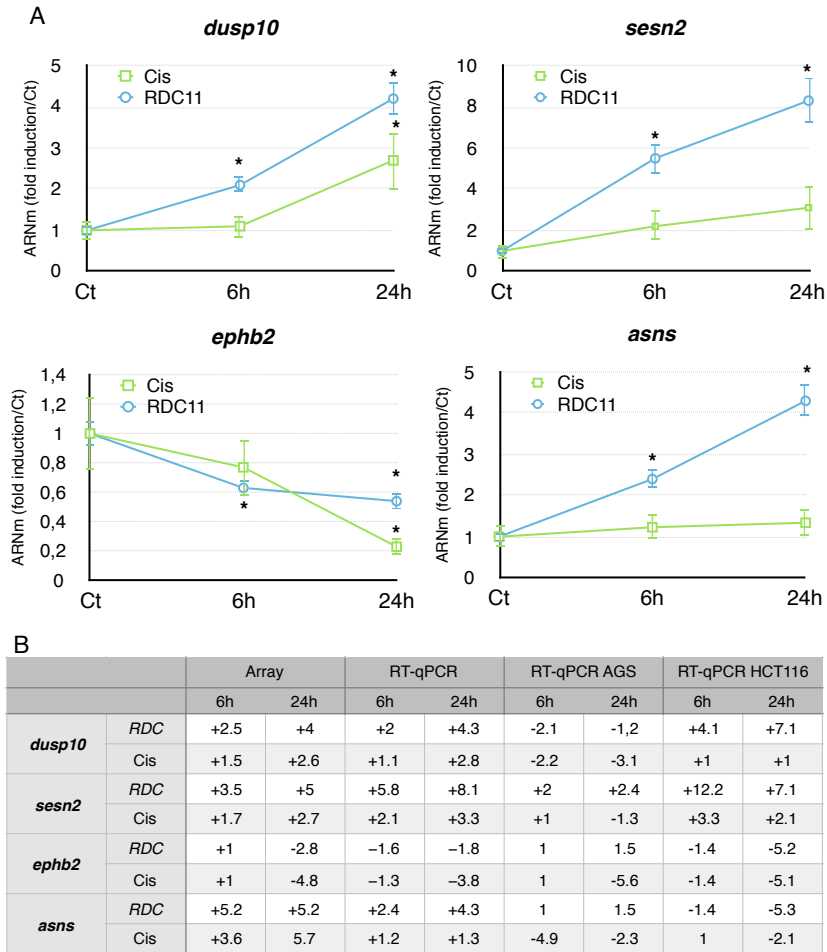
A

B		Array		RT-qPCR		RT-qPCR AGS		RT-qPCR HCT116	
		6h	24h	6h	24h	6h	24h	6h	24h
dusp10	RDC	+2.5	+4	+2	+4.3	-2.1	-1,2	+4.1	+7.1
	Cis	+1.5	+2.6	+1.1	+2.8	-2.2	-3.1	+1	+1
sesn2	RDC	+3.5	+5	+5.8	+8.1	+2	+2.4	+12.2	+7.1
	Cis	+1.7	+2.7	+2.1	+3.3	+1	-1.3	+3.3	+2.1
ephb2	RDC	+1	-2.8	−1.6	−1.8	1	1.5	-1.4	-5.2
	Cis	+1	-4.8	−1.3	−3.8	1	-5.6	-1.4	-5.1
asns	RDC	+5.2	+5.2	+2.4	+4.3	1	1.5	-1.4	-5.3
	Cis	+3.6	5.7	+1.2	+1.3	-4.9	-2.3	1	-2.1

mRNA levels of *genes found disregulated in the microarraus:* A. *dusp10*, *sesn2*, *eph2*, *asnas* were assayed in U87 (A, B RT-qPCR) colon cancer HCT116 (B) and gastric cancer AGS (B) cells by RT-qPCR. Curves are means of fold induction versus the control (Ct) with SD (n=3). *: p < 0.01. In B, number indicate average of fold changes.

Supplementary experimental procedures

Statistical analysis of Microarray data

Data obtained from Affymetrix hugene10stv1 arrays (http://www.ncbi.nlm.nih.gov / geo / query / acc.cgi?acc=GSE66493) were analyzed in R version 3.0.1. Raw data were first extracted from the CEL files and visualized using the package affyPLM to detect potential hybridization artifacts. Probeset annotations were retrieved from Ensembl (GRCh37.p10 release 69) with Biomart (BMC Genomics 2009, 10:22 doi: 10.1186 / 1471-2164-10-22) for 32325 probe-set corresponding to 25489 mapped genes. Normalization was performed either with RMA model from the affy package (Bioinformatics. 2004 Feb 12;20(3):307-15.) or with probe-level linear models (affyPLM) (Bioinformatics. 2004;20:307–315). The normalization methods were compared by plotting ROC curves using the respective adjusted p-values values as predictor. Differential expression analysis was performed with the limma package (BMC Genomics. 2006 Oct 9;7:252. Clustering was performed on a selection of 4540 probsets showing at least one condition with |FC| ≥1.5 and adjusted p-value ≤ 0.05. Hierarchical clustering was carried out with Pearson's correlation and the complete-linkage method. Clusters were defined by 1.5 times the height of the longest branch. Soft clustering was performed with the package Mfuzz using the parameters c=8 and m=1.6 (30). Gene Set enrichment analysis was carried out with MetaCore (Thomson Reuters) using human annotations, p-values ≤ 0.05 were considered as significant. Pathway analysis and transcription factor target analysis were performed with AltAnalysis.

RDC11-matrix synthesis:

The RDC11 containing support used for affinity chromatography was obtained by coordinating the hypogel-400-CO(-5-amino-1,10-phenanthroline) resin to a coordinatively-unsaturated ruthenium derivative containing a cycloruthenated phenyl pyridine ligand.

1. Synthesis of the phenanthroline containing support.

Commercially available (Interchim) Hypogel-400-COOH (1g, 0.3mmol) and hydroxybenzotriazole (HOBt) 0.268g, 2mmol) were added in DMF/ CH_2Cl_2 (2:1, 15 mL). To this solution EDCI.HCl (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl) (0.379g, 2mmol) and diisopropylethylamine (60 µL (microL), 4 mmol) were added. After 30 min. stirring, 5-amino-1,10-phenanthroline (0.129g, 0.66 mmol) suspended in DMF was added and the reaction mixture was stirred for 24h at room temperature. The resulting solid support was filtered and washed consecutively with DMF/CH_2Cl_2 (5/5 mL), CH_2Cl_2 (10 mL) and Et_2O (10 mL). After drying in high vacuum the modified resin was used as such for the next step.

2. Synthesis of the RDC11 containing support.

The modified support obtained above (0.5g, 1.0 eq.) was suspended in dry CH_2Cl_2 (10 mL) to which was added [Ru(2-phenyl-2'-pyridine) (MeCN)$_4$]PF$_6$ (50.028 mg, 1 eq.) [ref]. The reaction mixture was stirred for 24h at room temperature. The black solid support was filtered and washed consecutively with DMF/ CH_2Cl_2, CH_2Cl_2 and Et_2O. Drying this solid under high vacuum afforded the required RDC11-containing support.

ref: Fernandez, S.; Pfeffer, M.; Ritleng, V.; Sirlin, C. Organometallics 1999, 18, 2390-2394.
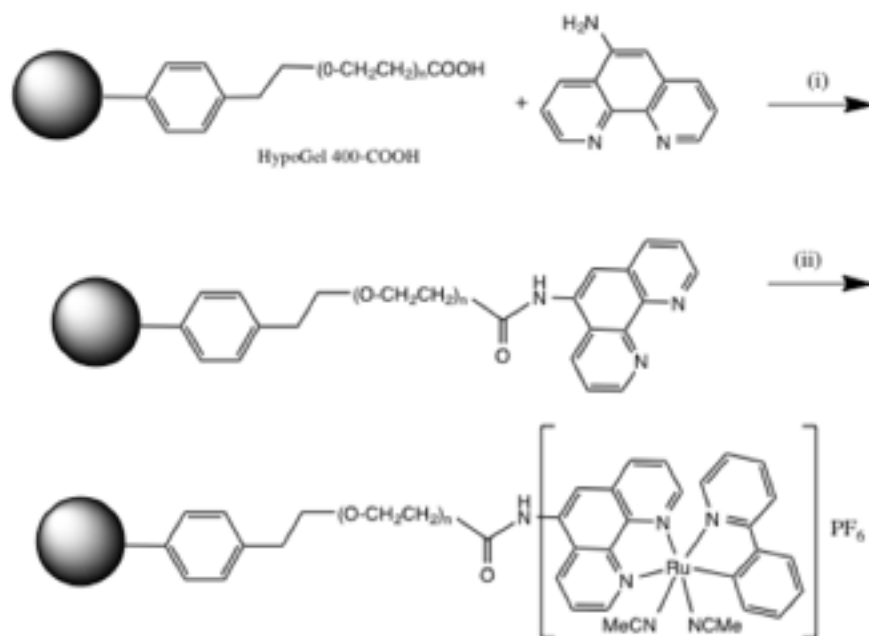
Digestion

In gel digestion

Stained protein bands were excised prior to destaining, in-gel reduction and alkylation of proteins, which were performed using a MassPREP Station (Waters, Manchester, UK). Briefly, destaining was done by three washes in a mixture containing 25 mM NH_4HCO_3:CH_3CN (1:1, v:v) followed by 30 min of dehydration in acetonitrile at 60°C for 5 min. Cysteine residues were reduced by 50 µL of 10 mM dithiothreitol, 25mM NH_4HCO_3 at 57°C for 30 min and alkylated by 50 µL of 55 mM iodoacetamide, 25mM NH_4HCO_3 for 30 min. After washing with 50 µL of 25 mM NH_4HCO_3, dehydration was done with acetonitrile during 15 min. Proteins were cleaved in-gel using 40 µL of 12.5 ng/µL of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at 37°C for 4 hours. Tryptic peptides were first extracted using a 60% acetonitrile solution containing 0.5% formic acid, then secondly with a 100% acetonitrile solution.

Liquid digestion

Sample were dried and solubilized in 200 µl of 8 Murea in 100 mM NH4HCO3 buffer. Each sample was reduced for 1 h at 60 °C by adding dithiothreitol to a final concentration of 10 mM. Alkylation was performed by adding iodoacetamide to a final concentration of 40 mM, 1 h at room temperature. To carry out the digestion in an optimal way, the sample was diluted to 1 M urea. An overnight digestion was performed by adding trypsin in a 1:50 enzyme to protein ratio. All samples were desalted on Sep-Pak C18 cartridges.

LC-MS/MS and data analysis

NanoLC-MS/MS analyses were performed on two systems : i/ a nanoACQUITY Ultra-Performance-LC system (UPLC) coupled to a SYNAPT HDMS Q-TOF mass spectrometer equipped with a nano-electrospray source (Waters), ii/ an Agilent 1100 series nanoLC-Chip/MS system (Agilent Technologies, Palo Alto, USA) coupled to an ion trap (amaZon, Bruker Daltonics, Bremen, Germany).

<u>i/ NanoACQUITY Ultra-Performance-LC system coupled to a </u>SYNAPT HDMS Q-TOF

nanoACQUITY UPLC system and SYNAPT HDMS Q-TOF mass spectrometer were controlled by MassLynx v4.1 (SCN 566, Waters). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). Peptides were injected and first trapped during 3 min on a precolumn (Symmetry C18, 20 mm x 0.18 mm, 5 µm particle size, Waters) at a flow rate of 5 µL/min with 99% A, then eluted at 45°C on a separation column (ACQUITY UPLC® BEH130 C18, 200mm x 75 µm, 1.7 µm particle size, Waters) at a flow rate of 400 nL/min using a 35 min linear gradient from 1 to 40% B and followed by 5 min at 65% B. The mass spectrometer was operating in positive mode with the following settings: source temperature was set to 80°C, cone gas flow was 30l/h, cone voltage was 40V and the nanoelectrospray voltage was optimized to 3.5kV. Mass calibration of the TOF was achieved using phosphoric acid (H_3PO_4) on the 50-2000 m/z range. Online correction of this calibration was done using product ions derived from the [Glu1]-fibrinopeptide B (GFP) as lock-mass compounds. The ion $(M+2H)^{2+}$ at m/z 785.8426 was used to calibrate MS data and the fragment ion $(M+H)^+$ at m/z 684.3469 to calibrate MS/MS data.

Spectra were acquired by automatic switching between MS and MS/MS modes. This was done in the mass range of 250-1500 m/z (MS, 0.5 sec) and 50-2000 m/z (MS/MS, 0.7sec). The most abundant peptide ions (the 3 most intense with a threshold of 60 counts/sec), preferably with a charge of 2 to 4, were selected from each MS spectrum for further isolation and CID (Collision Induced Dissociation) fragmentation using argon as collision gas. Ions were excluded after acquisition of one MS/MS spectrum and exclusion was released after 0.6 min. NanoLC-MS/MS raw data generated were respectively converted into « .pkl » peaklists with PLGS 2.3 (Waters).

<u>ii/ Agilent 1100 series nanoLC-Chip/MS system coupled to an amaZon ion trap</u>

This system was fully controlled by HyStar 3.2 (BrukerDaltonics). The chip contained a Zorbax 300SB-C18 column (43 mm×75 µm, 5 µm particle size) and a Zorbax 300SB-C18 enrichment column (40 nL, 5 µm particle size). The solvent system consisted of 2% acetonitrile, 0.1% formic acid in water (solvent A) and 2% water, 0.1% formic acid in acetonitrile (solvent B).

3 µl of each sample were loaded into the enrichment column at a flow rate set to 3.75 µL/min with solvent A. Elution was performed at a flow rate of 300 nL/min with a 8–40% linear gradient (solvent B) in 30 min (in gel) or 45/90 min (liquid sample, short/long gradient), followed by a 4 min stage at 70% of solvent B before reconditioning the column at 8% of solvent B.

MS spectrums were acquired with the following settings: source temperature was set to 135°C while cone gas flow was at 3 L/min. The nanoelectrospray voltage was optimized to −1850 V. The MS spectra were acquired in the positive ion mode on the mass range 250 to 1500 m/z using the standard enhanced resolution at a scan rate of 8.1 m/z/s. The Ion Charge Control was fixed at 200000 with a maximum accumulation time of 200 ms. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. Fragmentation was performed using argon as the collision gas. The 6 most abundant peptides were selected on each MS spectrum for further isolation and fragmentation with a preference for doubly charged ions (absolute threshold of 5000, relative threshold 5%). Ions were excluded after the acquisition of 1 MS/MS spectra and the exclusion was released after 0.3 min. The Smart Parameters Setting option was used for the selected precursor ions. The MS/MS spectra were acquired on the mass range 100 to 2000 m/z. The Ion Charge Control was fixed at 300000 and 2 scans were averaged to obtain a MS/MS spectrum.

The system was fully controlled by HyStar 3.2 (BrukerDaltonics). Mass data collected during the nanoLC-MS/MS analyses were processed and converted into .mgf files using the DataAnalysis 3.3 Build 146 software (Bruker Daltonics).

Protein identification

Peaklists (.pkl and .mgf) were searched using a local Mascot server (version 2. 2. 0, MatrixScience, London, UK) against a combined target-decoy protein database containing protein sequences of *Homo sapiens* (taxonomy 9606) derived from UniprotKB (created 2015-10-30, 84456 entries) and common contaminants such as human keratins and trypsin. The database was created using an in-house database generation toolbox (Carapito and al. 2014). Database searches were performed using the following settings: trypsin was specified as enzyme and up to one missed cleavage by trypsin and three variable modifications (oxidation of methionine, carbamidomethylation of cysteine and acetylation of protein N-termini) were considered. Mass tolerances on precursor and fragment ions were set to 20 ppm and 0.2 Da, respectively for system 1 and 50 ppm and 0.2 Da for system 2. Mass tolerances on precursor and fragment ions were set to 25 ppm and 0.07 Da for SYNAPT and 250 ppm and 0.5 Da for Amazon.

Identifications were validated with the in house developed Proline software (http://proline.profiproteomics.fr/) using the following validation criteria: target/decoy validation applying a 1% FDR at protein level.

Carapito and al. 2014
Proteomics. 2014 May;14(9):1014-9. doi: 10.1002/pmic.201300415. Epub 2014 Mar 12. MSDA, a proteomics software suite for in-depth Mass Spectrometry Data Analysis using grid computing. Carapito C, Burel A, Guterl P, Walter A, Varrier F, Bertile F, Van Dosselaer A.

VII. DISCUSSION / PERSPECTIVES

Gastric cancer has a high incidence in Europe, corresponding in majority of cardia cancer types, which are more aggressive and difficult to treat *(Pacelli and al., 2001). This type of cancer is increasing in France and presents a clinical problem with 4500 deaths / year due in part to the lack of efficacy of current treatments.* Surgical resection combined with chemotherapy based on organometallic compounds derived from platinum (cisplatin, oxaliplatin) are the cornerstone of current treatments *(Coccolini and al., 2016).* Unfortunately, only a limited number of tumor responds to the treatment. Resistance mechanisms of gastric tumors are not currently known, highlighting the need to identify robust molecular markers. The identification of these markers allows us firstly to classify the GC and adapt the treatment, and secondly, to develop targeted therapies. *This identification of markers and the advances in treatment require a better knowledge of the mechanisms of action of therapies and tumor biology possibly leading to more agressive cancers.*

Therefore, in order to develop more effective therapies, it is important to identify precisely what are the pathways deregulated by common and developed chemotherapy in the digestive system. Through the identification of new relay in the biology of GC, this work may improve patients care and allows the optimization of treatment. During my PhD, I had initiated this project, which has two major aspects. First, I worked to better understand the mechanisms of action of cytotoxic drugs (cisplatin, RDC11, HDACi) and secondly, I was interested in the development of innovative treatments (combination of drugs, RDC11, HDACi) in the GC.

1. Epigenetic and chemotherapy response in gastric cancer

Defining the mode of action of anticancer drugs for their use in clinic is crucial. Ultimately, this would help to achieve so-called personalized therapies, but also the identification of new molecular markers to follow the treatment response and its effectiveness. For this, we combined transcriptome analysis with sequencing of the small RNA libraries in response to cytotoxic drugs (cisplatin, RDC11) that revealed precious information in terms of signaling pathways specifically the epigenetic pathway.

1.1. HDAC implication in cisplatin response

A microarray analysis allowed us to highlight the role of HDAC4 in cisplatin response. Indeed, we could implicate HDAC4 in GC resistance to cisplatin-based chemotherapy, by making two observations. The first is that HDAC4 expression is elevated in biopsies of GC compared to the normal tissue. The second is that the silencing of HDAC4 favors cisplatin cytotoxicity. In this context, it is necessary to better characterize the role of HDAC4 in GC by studying other aspects than *in vitro* cell survival. In fact, the role of HDAC4 in this process is significant but not major. It is possible that its role is more important for the survival of cancer cells in their low tumor oxygen environment in view of its target genes such as the hypoxia factor, HIF1α *(Hutt and al., 2014; Geng and al., 2011)*. It would be interesting to study how loss of function of HDAC4 impact on the biological activities of HIF1α, such as the regulation of cell metabolism, angiogenesis or migration. Ideally, this would require to inhibit the function of HDAC4 *in vivo*, either by performing a stable HDAC4 knockdown or knockout or by using a specific HDAC4 inhibitor.

In this context, the specific inhibition of HDAC4 by a HDACi could improve cancer therapies efficiency. Unfortunately, there is currently no efficient inhibitor of HDAC4. Our choice was therefore focused on the SAHA molecule. SAHA is an inhibitor of class I, II and IV of HDACs *(Kelly and al., 2005).* In view of the lack of treatment efficacy by HDACi used alone and the importance of platinum-derivatives side effects, I worked in the development of combined therapy with these two compounds. The use of combined treatments allows us to impact on several regulatory pathways simultaneously and potentiate the effect of each drug with lower concentrations. My results allowed to highlight the strong synergy of these combined treatments in GC, as previously reported *(Shen and al., 2007; Sato and al., 2006b; Suzuki and al., 2009b; Zhou and al., 2014; Yoo and al., 2014)*, but also to inform us about the mode of action involved in this response. The ability to use lower concentrations for each drug has a real interest regarding in their use in clinic, especially to reduce side effects, which represent an obstacle in the treatment. However, the use of SAHA, a pan-inhibitor present limits. Indeed, each HDACi has a greater affinity for one or another HDACs. Thus the level of expression of HDAC will dictate the sensitivity of tumors to treatment *(Ropero and al., 2007)*. It is important today to encourage the development of specific inhibitor of HDAC, to contribute to the overall effort in order to achieve a personalized therapy for patients.

To optimize GC treatment, we included in our study new HDAC inhibitors targeting specific class of HDAC. Among them, we tested the JAHA molecule, an analog of SAHA organometallic compound, which contains a ferrocene moiety, a specific classe I inhibitor *(Spencer and al., 2011; Librizzi and al., 2012)* and the LMK-235 inhibiting specifically HDAC4 and HDAC5 *(Marek and al., 2013)*. Our preliminary results show, as with the SAHA, a synergistic effect in the combination with the platinum derivatives.

It should however be mentioned here that these results were obtained *in vitro*, so it now remains to determine whether the same results are observed *in vivo*. These initial results are encouraging for the development of new therapies, which I hope will one day have the chance to enter clinical trials within the GC and perhaps may be approved later.

1.2. miRNA deregulated in response to anticancer drugs

I used a second unbiased approach, based on small RNA cloning and sequencing, to identify the potential implication of miRNAs in response to cytotoxic drugs in GC. This approach allowed us to identify more than 100 miRNAs deregulated in response to cisplatin and RDC11. However, we could not observe major differences in the expression of miRNAs between the two drugs.

Subsequently, I focused in more details on Let-7e, a known tumor suppressor *(Trang and al., 2011)*, and miR-183, a miRNA, which involvement in the response of GC to cisplatin is currently controversial *(Zhang and al., 2013b; Cao and al., 2014; Tang and al., 2013; Gu and al., 2014)*. The functional analysis of these two miRNAs helped to highlight their role in cell survival in response to cisplatin. However, blocking their expression is not sufficient to protect the cells to the treatment. This result seems consistent in view of the number of miRNAs described in humans, currently over 2500 regulating more than 60% of the genome *(Kozomara and Griffiths-Jones, 2014)*. In both cases the implementation of compensatory mechanism by other miRNAs could explain this result.

Moreover, I was also interested in the importance of miR-140 in HDAC4 regulation in response to cisplatin. We did not observe increased levels of miR-140 in the miRNA library, which can be probably explained by its early expression in response to the treatment. My results are attempting to define the role played by miR-140 in the chemosensitivity of the GC in response to cisplatin, involving the repression of HDAC4. Moreover, our results are supported by Wang et al., which suggest that miR-140 and HDAC4 may contribute to drug resistance in osteosarcomas and colon

cancer cells *(Wang and al., 2014)*. Moreover, Kim et al. observed an overexpression of miR-140 in human GC samples correlated with the tumor chemosensitivity *(Kim and al., 2011)*.

Taken together these results lead to the identification of some miRNA implicated in the relay of the anti-cancer drugs response. Finally the identification of miRNA important for treatment efficacy, combined with the development of new therapies targeting miRNAs *(Krutzfeldt and al., 2005)* will improve patient treatment. Moreover, miRNA library data will enable us subsequently to complete our microarray data analysis to answer our main question, which is to better understand the mechanisms of action of these two drugs in GC.

2. RDC11, a therapy in development

Previous work in our team showed that RDC11 has a cytotoxic activity higher than platinum derivatives and is active on platinum-resistant cell lines. In vivo, it reduces tumor growth in several syngenic and xenografts tumor models. In addition, RDC11 is less sensitive to the mechanisms of cisplatin resistance. Studies on RDC11 mode of action have shown that this compound can induce cell death or growth arrest, by DNA dependent and independent mechanisms, including the reticulum stress pathway *(Gaiddon and al., 2005; Meng and al., 2009; Vidimar and al., 2012; Klajner and al., 2014)*.

RDC11 mode of action

The microarray data analysis allowed us to achieve a macroscopic validation of the differences between the mechanisms of action of cisplatin and RDC11. For example, a strong involvement of the p53 and epigenetic pahtways in response to cisplatin is observed, versus the oxidative and the reticulum stress for the RDC11. Furthermore, this approach confirmed our hypothesis that DNA was not a primary target for RDC *(Gaiddon and al., 2005; Meng and al., 2009; Vidimar and al., 2012)*, and that mechanisms involved in cellular metabolism were more implicated in RDC11 in comparison with cisplatin.

The transcriptome analyses as well as a survey of the literature suggest that besides the classical apoptosis and cell cycle arrest processes, other mechanisms and signaling pathways could be induced or regulated by our family of metal-based compounds. For example, during my Ph.D, I obtained some key results that indicated a possible relation of RDC inducing the transulfuration pathway. This metabolic pathway impacts cell survival through glutathione, H2S, and cysteine synthesis. Interestingly, it is over-activated in cancer cells through the over-expression of some

implicated enzymes *(Ostrakhovitch and al., 2016, Zhang and al., 2016)*. Glutathione synthesis is dependent of different enzymes, such as CBS (Cystathionine Beta-Synthase), CTH (Cystathionine gamma-lyase) and the GCL (γ-glutamylcysteine Ligase) *(Hine et al., 2015, Hishiki et al., 2012)*. These reactions generate H2S which is responsible for the production of NAMPT (Nicotinamide phosphoribosyltransferase), a molecule which in turn increases the expression of CBS and CTH via an auto-activation loop *(Ostrakhovitch et al. 2016; Sanokawa- Akakura et al., 2014)* **(Figure 24)**. The impact of this pathway on cell survival, and especially of cancer cells, is still controversial. For example, the H2S can have opposite effects. On one hand, high levels of H2S can be toxic to cancer cells. On the other hand, it appears to have a protective effect against various stresses by participating in the regulation of the mitochondrial metabolism *(Zhang et al., 2016, Zong et al., 2016)*. In addition, this pathway is also responsible for the de novo synthesis of glutathione, which allows homeostasis maintenance of the redox potential in the cell and thereby prevents the apoptosis of cancer cells *(Mosharov et al., 2000)*.
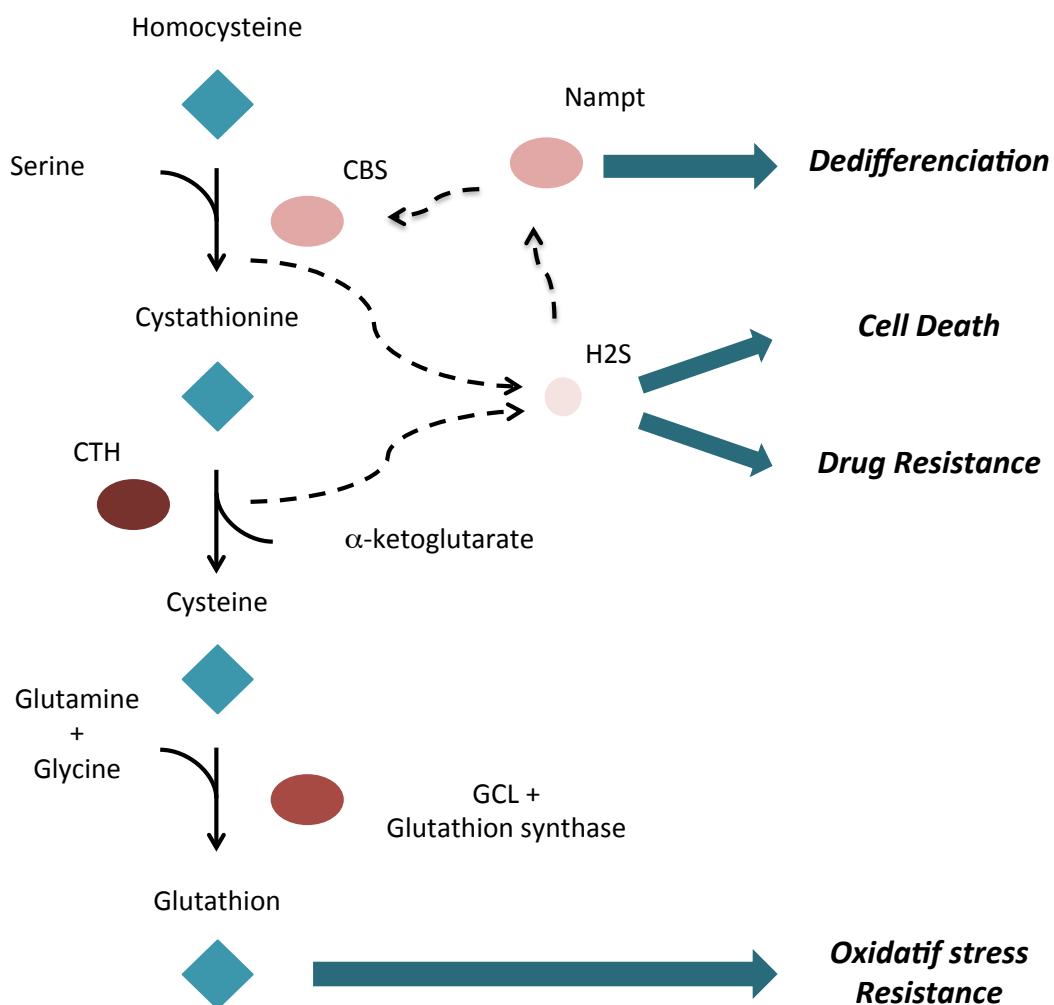

Figure 24: Schematic representation of the transulfuration pathway

I confirmed the relationship between RDC11 and the transulfuration pathway in GC cell lines. In fact, I showed that CTH, a crucial enzyme in this pathway, is strongly over-expressed (RNA and protein levels) in response to RDC11. However, several questions remain open. We must now determine how this pathway is regulated by RDC11 to better understand the molecular mechanisms implicated, especially how CTH contributes to the RDC-induced cytotoxicity. The work hypothesis, supported by results obtained by a new Ph.D student in our laboratory, is that this regulation is done through the reticulum stress pathway (UPR) by ATF4. In agreement with this hypothesis, Mistry and colleagues described that ATF4 regulates the CTH expression by a direct binding to its promoter in response to the UPR activation *(Mistry et al., 2015)*.

Activation of ATF4 by RDC11 suggests, in view of previous results obtained in the laboratory *(Bergamo and al., 2012; Vidimar and al., 2012)* that the RDC11 is able to activate the three parts of the UPR pathway (PERK, ATF6 and IRE1, **Figure 13**). This is not the case of platinum derivatives currently used in clinic, which confirms a dissimilarity between the mechanisms of action of these compounds. Finally, it will be important to identify more generally the role of the transulfuration pathway in the tumor aggressiveness. Indeed, it is a metabolic pathway that should also allow cells to adapt to their environment. It will thus be important to understand how the transulfuration pathway contributes to tumor growth by studying its role in the adaptation of cancer cells to hypoxia or metastasis.

Like I already described, our results show the importance of the reticulum stress pathway in response to RDC11. So, I looked in the literature for miRNAs that may be involved / regulated in this pathway, and I correlated this information with data from our miRNA library. MiRNAs are described as key regulators of homeostasis of the endoplasmic reticulum and are involved in the reticulum stress pathway *(Maurel and al., 2013; Byrd and al., 2013; Malhi and al., 2014)*. Indeed, the cluster miR-23a / 27a / 24-2 has been described to induce the expression of certain effector protein of this pathway, such as CHOP, TRIB3, ATF3 and ATF4, leading to cell apoptosis *(Chhabra and al., 2011)*. In addition, Chhabra and colleagues demonstrated that induction of this cluster expression leads to a caspase-dependent and independent apoptosis via JNK. This results in ROS generation allowing the activation of PERK and IRE1 pathways *(Chhabra and al., 2009)*. Furthermore, inhibition of miR-17 expression dependent of IRE1 and JNK allows the induction of TXNP expression (thioredoxin-interacting protoein) leading to cell apoptosis *(Lerner and al., 2012)*. Interestingly, we observed that the expression of miR-23a and miR-27a is strongly induced in response to RDC11, whereas the expression of miR-17 is repressed. The analysis of miRNAs

involved in the response to the RDC11 through the reticulum stress opens up new perspectives of study.

Finally, our study represents the first demonstration of a direct target for RDC11 by showing its interaction with histones. It also shows that ruthenium compounds have an impact on the post-translational modifications of histones. These modifications are part of complex and essential epigenetic mechanisms that are the control of gene expression. Indeed, our results show the possible involvement of HDACs in the biological activity of RDC11. So in this context, we tried to set up a combined therapy of RDC11 and SAHA. Surprisingly, the results showed an antagonistic effect of the combination of the RDC and SAHA. This result strongly suggest that HDAC inhibitors should not be used for a combinatory therapy in order to potentiate the cytotoxicity of RDC.

This work thus opens up new perspectives for understanding mechanisms of action of anticancer drugs, including gastric cancer, where this type of study has not been performed yet. However, the issues that remain unresolved are now to define if these signaling pathways are directly regulated by the RDC and cisplatin or are a consequence of adaptation / compensation mechanisms in response to other previous pathways activated. We now have to make the connection between all these activated signaling pathways, the stress reticulum, the transulfuration and the epigenetic pathways to better understand the mechanisms involved in RDC11 cytotoxicity against GC cells.

VIII.CONCLUSION

The work done during my PhD allowed the identification of epigenetic mechanisms important for the anticancer drugs response in GC, such as HDAC4 and miR140. In particular, I showed that HDAC4 inhibits the activity of cisplatin and its expression is particularly high in GC, making it a good candidate as a resistance mechanism. Moreover, I showed how these epigenetic regulation interact closely with members of the p53 family to control cell fate.

Using my results showing the crucial roles of p53 and HDAC in the therapeutic response, I participated in pre-clinical development of new anticancer compounds. In particular, I have shown that ruthenium compounds having redox properties act independently of the DNA and p53 but affect certain epigenetic regulations. Moreover, I have shown that cisplatin activity is greatly increased when combined with HDAC inhibitors.

Two PhD students will try to answer our open questions. I am pleased that this research project will continue.

IX. BIBLIOGRAPHY

Adimoolam, S. & Ford, J. M. p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proc. Natl. Acad. Sci. U. S. A. 99, 12985–90 (2002).

Adimoolam, S. & Ford, J. M. p53 and regulation of DNA damage recognition during nucleotide excision repair. DNA Repair (Amst). 2, 947–954 (2003).

Agami, R., Blandino, G., Oren, M. & Shaul, Y. Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. Nature 399, 809–13 (1999).

Aird, R. E. et al. In vitro and in vivo activity and cross resistance profiles of novel ruthenium (II) organometallic arene complexes in human ovarian cancer. Br. J. Cancer 86, 1652–7 (2002).

Akiyoshi, S. et al. Clinical significance of miR-144-ZFX axis in disseminated tumour cells in bone marrow in gastric cancer cases. Br. J. Cancer 107, 1345–53 (2012).

Al-Majed, A. A. Carnitine deficiency provokes cisplatin-induced hepatotoxicity in rats. Basic Clin. Pharmacol. Toxicol. 100, 145–50 (2007).

Alberts, S. R., Cervantes, A. & van de Velde, C. J. H. Gastric cancer: epidemiology, pathology and treatment. Ann. Oncol. 14 Suppl 2, ii31–6 (2003).

Alessio, E., Mestroni, G., Bergamo, A. & Sava, G. Ruthenium antimetastatic agents. Curr. Top. Med. Chem. 4, 1525–35 (2004).

Ali, I., Wani, W. A., Saleem, K. & Haque, A. Platinum compounds: a hope for future cancer chemotherapy. Anticancer. Agents Med. Chem. 13, 296–306 (2013).

ALLFREY, V. G., FAULKNER, R. & MIRSKY, A. E. ACETYLATION AND METHYLATION OF HISTONES AND THEIR POSSIBLE ROLE IN THE REGULATION OF RNA SYNTHESIS. Proc. Natl. Acad. Sci. U. S. A. 51, 786–94 (1964).

Allocati, N., Di Ilio, C. & De Laurenzi, V. p63/p73 in the control of cell cycle and cell death. Exp. Cell Res. 318, 1285–1290 (2012).

American cancer society, The stomach.

Andreucci, J. J. et al. Composition and function of AP-1 transcription complexes during muscle cell differentiation. J. Biol. Chem. 277, 16426–32 (2002).

Antonarakis, E. S. & Emadi, A. Ruthenium-based chemotherapeutics: are they ready for prime time? Cancer Chemother. Pharmacol. 66, 1–9 (2010).

Argyriou, A. A. et al. A review on oxaliplatin-induced peripheral nerve damage. Cancer Treat. Rev. 34, 368–377 (2008).

Ashburner, B. P., Westerheide, S. D. & Baldwin, A. S. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol. Cell. Biol. 21, 7065–77 (2001).

Asher, G., Tsvetkov, P., Kahana, C. & Shaul, Y. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. 19, 316–21 (2005).

Babar, I. A. et al. Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. Proc. Natl. Acad. Sci. U. S. A. 109, E1695–704 (2012).

Bachman, K. E. et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell 3, 89–95 (2003).

Bäckström, A. et al. Metastability of Helicobacter pylori bab adhesin genes and dynamics in Lewis b antigen binding. Proc. Natl. Acad. Sci. U. S. A. 101, 16923–8 (2004).

Bai, S. et al. DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2. Mol. Cell. Biol. 25, 751–66 (2005).

Bali, P. et al. Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. J. Biol. Chem. 280, 26729–34 (2005).

Bang, Y.-J. et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet (London, England) 376, 687–97 (2010).

Bartel, D. P. et al. MicroRNAs: target recognition and regulatory functions. Cell 136, 215–33 (2009).

Bartel, D. P. et al. MicroRNAs. Cell 116, 281–297 (2004).

Basu, A. & Krishnamurthy, S. Cellular responses to Cisplatin-induced DNA damage. J. Nucleic Acids 2010, (2010).

Bazzini, A. A., Lee, M. T. & Giraldez, A. J. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science 336, 233–7 (2012).

Beitzinger, M., Oswald, C., Beinoraviciute-Kellner, R. & Stiewe, T. Regulation of telomerase activity by the p53 family member p73. Oncogene 25, 813–26 (2006).

Bellacosa, A. Functional interactions and signaling properties of mammalian DNA mismatch repair proteins. Cell Death Differ. 8, 1076–92 (2001).

Bergamo, A. & Sava, G. Ruthenium complexes can target determinants of tumour malignancy. Dalton Trans. 1267–72 (2007). doi:10.1039/b617769g

Bergamo, A., Gaiddon, C., Schellens, J. H. M., Beijnen, J. H. & Sava, G. Approaching tumour therapy beyond platinum drugs: Status of the art and perspectives of ruthenium drug candidates. J. Inorg. Biochem. 106, 90–99 (2012).

Bergamo, A., Masi, A., Dyson, P. J. & Sava, G. Modulation of the metastatic progression of breast cancer with an organometallic ruthenium compound. Int. J. Oncol. 33, 1281–9 (2008).

Bergamo, A. & Sava, G. Ruthenium anticancer compounds: myths and realities of the emerging metal-based drugs. Dalton Trans. 40, 7817–23 (2011).

Berlth, F., Bollschweiler, E., Drebber, U., Hoelscher, A. H. & Moenig, S. Pathohistological classification systems in gastric cancer: diagnostic relevance and prognostic value. World J. Gastroenterol. 20, 5679–84 (2014).

Bernstein, B. E. et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125, 315–26 (2006).

Bertheau, P. et al. TP53 status and response to chemotherapy in breast cancer. Pathobiology 75, 132–9 (2008).

Bertos, N. R., Wang, A. H. & Yang, X. J. Class II histone deacetylases: structure, function, and regulation. Biochem. Cell Biol. 79, 243–52 (2001).

Bhaskara, S. et al. Hdac3 is essential for the maintenance of chromatin structure and genome stability. Cancer Cell 18, 436–47 (2010).

Biondi, A. & Hyung, W. J. Seventh edition of TNM classification for gastric cancer. J. Clin. Oncol. 29, 4338–9; author reply 4340–2 (2011).

Bird, A. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6–21 (2002).

Bjelakovic, G. et al. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. Lancet 364, 1219–1228 (2004).

Bode, A. M. & Dong, Z. Post-translational modification of p53 in tumorigenesis. Nat. Rev. Cancer 4, 793–805 (2004).

Bohnsack, M. T., Czaplinski, K. & Gorlich, D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 10, 185–91 (2004).

Boominathan, L. The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. PLoS One 5, e10615 (2010).

Bos, J. L. ras oncogenes in human cancer: a review. Cancer Res. 49, 4682–9 (1989).

Boulikas T, Pantos A, Bellis E, Christofis P. Designing platinum compounds in cancer: Structures and mechanisms. Cancer Therapy, 5, 537-83 (2007b).

Boulikas, T. Molecular mechanisms of cisplatin and its liposomally encapsulated form, LipoplatinTM. LipoplatinTM as a chemotherapy and antiangiogenesis drug. Cancer Ther. Vol Cancer Ther. 5, 351–376 (2007a).

Bourdon, J.-C. et al. p53 isoforms can regulate p53 transcriptional activity. Genes Dev. 19, 2122–37 (2005).

Brabec, V. et al. Modifications of DNA by platinum complexes. Relation to resistance of tumors to platinum antitumor drugs. Drug Resist. Updat. 8, 131–46 (2005).

Brehm, A. et al. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391, 597–601 (1998).

Brooks, C. L. & Gu, W. The impact of acetylation and deacetylation on the p53 pathway. Protein Cell 2, 456–62 (2011).

Brown, M. K. & Naidoo, N. The endoplasmic reticulum stress response in aging and age-related diseases. Front. Physiol. 3, 263 (2012).

Bruno, T. et al. Che-1 promotes tumor cell survival by sustaining mutant p53 transcription and inhibiting DNA damage response activation. Cancer Cell 18, 122–34 (2010).

Burger, H. et al. Drug transporters of platinum-based anticancer agents and their clinical significance. Drug Resist. Updat. 14, 22–34 (2011).

Burk, U. et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 9, 582–9 (2008).

Buschmann, T. et al. Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol. Cell. Biol. 21, 2743–54 (2001).

Bushati, N. & Cohen, S. M. microRNA functions. Annu. Rev. Cell Dev. Biol. 23, 175–205 (2007).

Byrd, A. E. & Brewer, J. W. Micro(RNA)managing endoplasmic reticulum stress. IUBMB Life 65, 373–81 (2013).

Caldas, C. et al. Familial gastric cancer: overview and guidelines for management. J. Med. Genet. 36, 873–80 (1999).

Calin, G. A. et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. U. S. A. 99, 15524–9 (2002).

Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513, 202–9 (2014).

Candi, E., Agostini, M., Melino, G. & Bernassola, F. How the TP53 family proteins TP63 and TP73 contribute to tumorigenesis: regulators and effectors. Hum. Mutat. 35, 702–14 (2014).

Cao, L.-L. et al. miR-183 inhibits invasion of gastric cancer by targeting Ezrin. Int. J. Clin. Exp. Pathol. 7, 5582–94 (2014).

Carl-Mcgrath, S., Ebert, M. & Röcken, C. Gastric adenocarcinoma: epidemiology, pathology and pathogenesis. Cancer Ther. Vol Cancer Ther. 5, 877–894 (2007).

Carter, S. K. et al. Carboplatin: future directions. Cancer Treat. Rev. 12, 145–152 (1985).

Casciano, I. et al. Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. Cell Death Differ. 9, 246–51 (2002).

Cedar, H. & Bergman, Y. Linking DNA methylation and histone modification: patterns and paradigms. Nat. Rev. Genet. 10, 295–304 (2009).

Celli, J. & Tsolis, R. M. Bacteria, the endoplasmic reticulum and the unfolded protein response: friends or foes? Nat. Rev. Microbiol. 13, 71–82 (2015).

Chakraborty, J. et al. Gain of cellular adaptation due to prolonged p53 impairment leads to functional switchover from p53 to p73 during DNA damage in acute myeloid leukemia cells. J. Biol. Chem. 285, 33104–12 (2010).

Chan, H. M. & La Thangue, N. B. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J. Cell Sci. 114, 2363–73 (2001).

Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W. & Vogelstein, B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature 401, 616–20 (1999).

Chang, B., Nishikawa, M., Sato, E., Utsumi, K. & Inoue, M. l-Carnitine inhibits cisplatin-induced injury of the kidney and small intestine. Arch. Biochem. Biophys. 405, 55–64 (2002).

Chang, C.-J. et al. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat. Cell Biol. 13, 317–23 (2011).

Chang, T.-C. et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell 26, 745–52 (2007).

Chatterjee, S., Kundu, S., Bhattacharyya, A., Hartinger, C. G. & Dyson, P. J. The ruthenium(II)-arene compound RAPTA-C induces apoptosis in EAC cells through mitochondrial and p53-JNK pathways. J. Biol. Inorg. Chem. 13, 1149–55 (2008).

Chen, D., Brooks, C. L. & Gu, W. ARF-BP1 as a potential therapeutic target. Br. J. Cancer 94, 1555–8 (2006).

Chen, H., Parkinson, J. A., Morris, R. E. & Sadler, P. J. Highly selective binding of organometallic ruthenium ethylenediamine complexes to nucleic acids: novel recognition mechanisms. J. Am. Chem. Soc. 125, 173–86 (2003).

Chen, H. et al. Organometallic ruthenium(II) diamine anticancer complexes: arene-nucleobase stacking and stereospecific hydrogen-bonding in guanine adducts. J. Am. Chem. Soc. 124, 3064–82 (2002).

Chen, Q. & Lesnefsky, E. J. Blockade of electron transport during ischemia preserves bcl-2 and inhibits opening of the mitochondrial permeability transition pore. FEBS Lett. 585, 921–6 (2011).

Chen, T., Xu, X.-Y. & Zhou, P.-H. Emerging molecular classifications and therapeutic implications for gastric cancer. Chin. J. Cancer 35, 49 (2016b).

Chen, X., Zheng, Y., Zhu, J., Jiang, J. & Wang, J. p73 is transcriptionally regulated by DNA damage, p53, and p73. Oncogene 20, 769–74 (2001).

Chen, Y.-C. et al. Clinicopathological Variation of Lauren Classification in Gastric Cancer. Pathol. Oncol. Res. 22, 197–202 (2016a).

Chendrimada, T. P. et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436, 740–4 (2005).

Chhabra, R., Adlakha, Y. K., Hariharan, M., Scaria, V. & Saini, N. Upregulation of miR-23a∼27a∼24-2 Cluster Induces Caspase-Dependent and -Independent Apoptosis in Human Embryonic Kidney Cells. PLoS One 4, e5848 (2009).

Chhabra, R., Dubey, R. & Saini, N. Gene expression profiling indicate role of ER stress in miR-23a~27a~24-2 cluster induced apoptosis in HEK293T cells. RNA Biol. 8, 648–664 (2011).

Chiang, Y. et al. Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. J. Gastrointest. Surg. 15, 63–70 (2011).

Chirino, Y. I. & Pedraza-Chaverri, J. Role of oxidative and nitrosative stress in cisplatin-induced nephrotoxicity. Exp. Toxicol. Pathol. 61, 223–242 (2009).

Cho, H. M. et al. microRNA-101 inhibits lung cancer invasion through the regulation of enhancer of zeste homolog 2. Exp. Ther. Med. 2, 963–967 (2011).

Cho, W. J. et al. miR-372 regulates cell cycle and apoptosis of ags human gastric cancer cell line through direct regulation of LATS2. Mol. Cells 28, 521–7 (2009).

Choi, J. H. et al. Expression profile of histone deacetylase 1 in gastric cancer tissues. Jpn. J. Cancer Res. 92, 1300–4 (2001).

Choudhary, C. et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325, 834–40 (2009).

Chuang, J. C. & Jones, P. A. Epigenetics and microRNAs. Pediatr. Res. 61, 24R–29R (2007).

Chueh, A. C., Tse, J. W. T., Tögel, L. & Mariadason, J. M. Mechanisms of Histone Deacetylase Inhibitor-Regulated Gene Expression in Cancer Cells. Antioxid. Redox Signal. 23, 66–84 (2015).

Clarke MJ. Ruthenium metallopharmaceuticals. Coordination Chemistry Reviews, 232(1), 69-93 (2002)

Coccolini, F. et al. Advanced gastric cancer: What we know and what we still have to learn. World J. Gastroenterol. 22, 1139–59 (2016).

Cohen, S. M. & Lippard, S. J. Cisplatin: from DNA damage to cancer chemotherapy. Prog. Nucleic Acid Res. Mol. Biol. 67, 93–130 (2001).

CORREA, P., Haenszel, W., Cuello, C., Tannenbaum, S. & Archer, M. A MODEL FOR GASTRIC CANCER EPIDEMIOLOGY. Lancet 306, 58–60 (1975).

Correa, P. & Shiao, Y. H. Phenotypic and genotypic events in gastric carcinogenesis. Cancer Res. 54, 1941s–1943s (1994).

Correa, P. et al. Carcinogenesis of Helicobacter pylori. Gastroenterology 133, 659–72 (2007).

Corso, G., Roncalli, F., Marrelli, D., Carneiro, F. & Roviello, F. History, pathogenesis, and management of familial gastric cancer: original study of John XXIII's family. Biomed Res. Int. 2013, 385132 (2013).

Costanzo, A. et al. DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. Mol. Cell 9, 175–86 (2002).

Coutandin, D. et al. Conformational stability and activity of p73 require a second helix in the tetramerization domain. Cell Death Differ. 16, 1582–9 (2009).

Cress, W. D. & Seto, E. Histone deacetylases, transcriptional control, and cancer. J. Cell. Physiol. 184, 1–16 (2000).

Crew, K. D. & Neugut, A. I. Epidemiology of gastric cancer. World J. Gastroenterol. 12, 354–62 (2006).

Creyghton, M. P. et al. H2AZ is enriched at polycomb complex target genes in ES cells and is necessary for lineage commitment. Cell 135, 649–61 (2008).

Cristescu, R. et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat. Med. 21, 449–56 (2015).

Crul, M. et al. DNA-based drug interactions of cisplatin. Cancer Treat. Rev. 28, 291–303 (2002).

Cullen, K. J., Yang, Z., Schumaker, L. & Guo, Z. Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer. J. Bioenerg. Biomembr. 39, 43–50 (2007).

d'Ydewalle, C., Bogaert, E. & Van Den Bosch, L. HDAC6 at the Intersection of Neuroprotection and Neurodegeneration. Traffic 13, 771–9 (2012).

Dai, J. M., Wang, Z. Y., Sun, D. C., Lin, R. X. & Wang, S. Q. SIRT1 interacts with p73 and suppresses p73-dependent transcriptional activity. J. Cell. Physiol. 210, 161–6 (2007).

Dalton, L. E. et al. The endoplasmic reticulum stress marker CHOP predicts survival in malignant mesothelioma. Br. J. Cancer 108, 1340–7 (2013).

Dang, C. V. et al. Function of the c-Myc Oncogenic Transcription Factor. Exp. Cell Res. 253, 63–77 (1999).

Dasari, S. & Bernard Tchounwou, P. Cisplatin in cancer therapy: Molecular mechanisms of action. Eur. J. Pharmacol. 740, 364–378 (2014).

Davison, T. S. et al. p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53. J. Biol. Chem. 274, 18709–14 (1999).

Davison, T. S. et al. Structure and functionality of a designed p53 dimer. J. Mol. Biol. 307, 605–617 (2001).

Deltour, S., Chopin, V. & Leprince, D. Modifications épigénétiques et cancer. M/S médecine Sci. 21, 405–411 (2005).

de Ruijter, A. J. M., van Gennip, A. H., Caron, H. N., Kemp, S. & van Kuilenburg, A. B. P. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370, 737–49 (2003).

Di Giorgio, E., Gagliostro, E. & Brancolini, C. Selective class IIa HDAC inhibitors: myth or reality. Cell. Mol. Life Sci. 72, 73–86 (2015).

Donehower, L. A. et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356, 215–21 (1992).

Egger, G., Liang, G., Aparicio, A. & Jones, P. A. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429, 457–63 (2004).

Eichhorn, S. W. et al. mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. Mol. Cell 56, 104–15 (2014).

El-Awady, E.-S. E., Moustafa, Y. M., Abo-Elmatty, D. M. & Radwan, A. Cisplatin-induced cardiotoxicity: Mechanisms and cardioprotective strategies. Eur. J. Pharmacol. 650, 335–341 (2011).

El-Deiry, W. S. Regulation ofp53downstream genes. Semin. Cancer Biol. 8, 345–357 (1998).

El-Omar, E. M. et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 404, 398–402 (2000).

El-Omar, E. M. et al. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology 124, 1193–1201 (2003).

Elmore, S. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516 (2007).

Esau, C. et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 3, 87–98 (2006).

Espada, J. et al. Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. J. Biol. Chem. 279, 37175–84 (2004).

Esteller, M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 21, 5427–40 (2002).

Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum Mol. Gen., 16, R50-R59 (2007).

Esteller, M. Epigenetics in Cancer. http://dx.doi.org/10.1056/NEJMra072067 (2009).

Fabian, M. R. & Sonenberg, N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat. Struct. Mol. Biol. 19, 586–93 (2012).

Fajas, L. et al. The Retinoblastoma-Histone Deacetylase 3 Complex Inhibits PPARγ and Adipocyte Differentiation. Dev. Cell 3, 903–910 (2002).

Feldman RA. Epidemiologic observations and open questions about disease and infection caused by Helicobacter pylori. In: Achtman M, Serbaum S. Helicobacter pylori: Molecular and Cellular Biology. Wymondham: Horizon Scientific,29-51, (2001).

Felsenfeld, G. & Groudine, M. Controlling the double helix. Nature 421, 448–453 (2003).

Fernandez, P. C. et al. Genomic targets of the human c-Myc protein. Genes Dev. 17, 1115–29 (2003).

Ferro, A. et al. Worldwide trends in gastric cancer mortality (1980-2011), with predictions to 2015, and incidence by subtype. Eur. J. Cancer 50, 1330–44 (2014).

Fetzer, L. et al. Library of second-generation cycloruthenated compounds and evaluation of their biological properties as potential anticancer drugs: passing the nanomolar barrier. Dalton Trans. 40, 8869–78 (2011).

Filion, G. & Defossez, P.-A. Les protéines se liant à l'ADN méthylé : interprètes du code épigénétique. M/S. Médecine Sci. [ISSN Pap. 0767-0974 ; ISSN numérique 1958-5381], 2004, Vol. 20, N° 1; p. 7-8

Fink, D. et al. The role of DNA mismatch repair in platinum drug resistance. Cancer Res. 56, 4881–6 (1996).

Fischer, D. D. et al. Isolation and characterization of a novel class II histone deacetylase, HDAC10. J. Biol. Chem. 277, 6656–66 (2002).

Fischle, W. et al. Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J. Biol. Chem. 276, 35826–35 (2001).

Fischle, W. et al. Enzymatic Activity Associated with Class II HDACs Is Dependent on a Multiprotein Complex Containing HDAC3 and SMRT/N-CoR. Mol. Cell 9, 45–57 (2002).

Fitch, M. E., Cross, I. V & Ford, J. M. p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, in vivo. Carcinogenesis 24, 843–50 (2003).

Fléjou, J.-F. Classification OMS 2010 des tumeurs digestives : la quatrième édition. Ann. Pathol. 31, S27–S31 (2011).

Florea, A.-M. & Büsselberg, D. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancers (Basel). 3, 1351–71 (2011).

Flores, E. R. et al. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell 7, 363–73 (2005).

Fontemaggi, G. et al. The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation. Mol. Cell. Biol. 21, 8461–70 (2001).

Forman, D. et al. Gastric cancer: global pattern of the disease and an overview of environmental risk factors. Best Pract. Res. Clin. Gastroenterol. 20, 633–649 (2006).

Fox, J. G. & Wang, T. C. Inflammation, atrophy, and gastric cancer. J. Clin. Invest. 117, 60–9 (2007).

Fraga, M. F. et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat. Genet. 37, 391–400 (2005).

Fridman, J. S. & Lowe, S. W. Control of apoptosis by p53. Oncogene 22, 9030–40 (2003).

Friedman, R. C., Farh, K. K.-H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105 (2009).

Fuchs, S. Y. et al. JNK targets p53 ubiquitination and degradation in nonstressed cells. Genes Dev. 12, 2658–63 (1998).

Fukagawa, T. et al. Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. 6, 784–91 (2004).

Fukazawa, T. et al. Differential involvement of the CD95 (Fas/APO-1) receptor/ligand system on apoptosis induced by the wild-type p53 gene transfer in human cancer cells. Oncogene 18, 2189–99 (1999).

Gaarz, A. et al. Bead array-based microrna expression profiling of peripheral blood and the impact of different RNA isolation approaches. J. Mol. Diagn. 12, 335–44 (2010).

Gaiddon, C. et al. Ruthenium (II)-derived organometallic compounds induce cytostatic and cytotoxic effects on mammalian cancer cell lines through p53-dependent and p53-independent mechanisms. J. Pharmacol. Exp. Ther. 315, 1403–11 (2005).

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T. & Prives, C. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol. Cell. Biol. 21, 1874–87 (2001).

Galanski, M., Arion, V. B., Jakupec, M. A. & Keppler, B. K. Recent developments in the field of tumor-inhibiting metal complexes. Curr. Pharm. Des. 9, 2078–89 (2003).

Galasinski, S. C., Resing, K. A., Goodrich, J. A. & Ahn, N. G. Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions. J. Biol. Chem. 277, 19618–26 (2002).

Gao, L., Cueto, M. A., Asselbergs, F. & Atadja, P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J. Biol. Chem. 277, 25748–55 (2002).

Gartel, A. L. & Radhakrishnan, S. K. Lost in transcription: p21 repression, mechanisms, and consequences. Cancer Res. 65, 3980–5 (2005).

Gava, B., Zorzet, S., Spessotto, P., Cocchietto, M. & Sava, G. Inhibition of B16 melanoma metastases with the ruthenium complex imidazolium trans-imidazoledimethylsulfoxide-tetrachlororuthenate and down-regulation of tumor cell invasion. J. Pharmacol. Exp. Ther. 317, 284–91 (2006).

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R. & Haas, R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. Science 301, 1099–102 (2003).

Geng, H. et al. HDAC4 protein regulates HIF1α protein lysine acetylation and cancer cell response to hypoxia. J. Biol. Chem. 286, 38095–102 (2011).

Gerhard, M. et al. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. Proc. Natl. Acad. Sci. U. S. A. 96, 12778–83 (1999).

Glass, C. K. & Rosenfeld, M. G. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121–41 (2000).

Glimelius, B. et al. Randomized comparison between chemotherapy plus best supportive care with best supportive care in advanced gastric cancer. Ann. Oncol. 8, 163–8 (1997).

Gong, J. G. et al. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 399, 806–9 (1999).

González, C. A. et al. Smoking and the risk of gastric cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). Int. J. cancer 107, 629–34 (2003).

Griffiths-Jones, S., Saini, H. K., van Dongen, S. & Enright, A. J. miRBase: tools for microRNA genomics. Nucleic Acids Res. 36, D154–8 (2008).

Grob, T. J. et al. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. Cell Death Differ. 8, 1213–23 (2001).

Gu, W. et al. Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p53 C-Terminal Domain. Cell 90, 595–606 (1997).

Gu, W. et al. MicroRNA-183 inhibits apoptosis and promotes proliferation and invasion of gastric cancer cells by targeting PDCD4. Int. J. Clin. Exp. Med. 7, 2519–29 (2014).

Guardiola, A. R. & Yao, T.-P. Molecular cloning and characterization of a novel histone deacetylase HDAC10. J. Biol. Chem. 277, 3350–6 (2002).

Guenther, M. G., Barak, O. & Lazar, M. A. The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol. Cell. Biol. 21, 6091–101 (2001).

Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. 15, 509–24 (2014).

Haberland, M., Montgomery, R. L. & Olson, E. N. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. 10, 32–42 (2009).

Hail, N., Carter, B. Z., Konopleva, M. & Andreeff, M. Apoptosis effector mechanisms: a requiem performed in different keys. Apoptosis 11, 889–904 (2006).

Halkidou, K. et al. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate 59, 177–89 (2004).

Hamilton, S. R. and Aalton, L. (eds.) Pathology and Genetics of Tumours of the Digestive System. IARC Press: Lyon Pathology and Genetics. Tumours of the Digestive System. WHO Classification of Tumours, Volume 2. IARC Press: Lyon (2000)

Han, J. et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125, 887–901 (2006).

Hanahan, D. et al. Hallmarks of cancer: the next generation. Cell 144, 646–74 (2011).

Hanke, M. et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. Urol. Oncol. 28, 655–61 (2006).

Harms, K., Nozell, S. & Chen, X. The common and distinct target genes of the p53 family transcription factors. Cell. Mol. Life Sci. 61, 822–42 (2004).

Harms, K. L. & Chen, X. Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. Cancer Res. 67, 3145–52 (2007).

Hartinger, C. G. et al. From bench to bedside – preclinical and early clinical development of the anticancer agent indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14A). J. Inorg. Biochem. 100, 891–904 (2006).

Hartmann, J. T. & Lipp, H.-P. Toxicity of platinum compounds. Expert Opin. Pharmacother. 4, 889–901 (2003).

Harvey, J. A. et al. Chemoradiation therapy is effective for the palliative treatment of malignant dysphagia. Dis. Esophagus 17, 260–5 (2004).

Hayakawa, Y., Sethi, N., Sepulveda, A. R., Bass, A. J. & Wang, T. C. Oesophageal adenocarcinoma and gastric cancer: should we mind the gap? Nat. Rev. Cancer 16, 305–18 (2016).

Hayes, J. et al. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol. Med. 20, 460–9 (2014).

Hayward, R. L. et al. Investigation of the role of Bax, p21/Waf1 and p53 as determinants of cellular responses in HCT116 colorectal cancer cells exposed to the novel cytotoxic ruthenium(II) organometallic agent, RM175. Cancer Chemother. Pharmacol. 55, 577–83 (2005).

He, L. et al. A microRNA polycistron as a potential human oncogene. Nature 435, 828–33 (2005).

He, L. et al. A microRNA component of the p53 tumour suppressor network. Nature 447, 1130–4 (2007).

He, Q., Liang, C. H. & Lippard, S. J. Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. Proc. Natl. Acad. Sci. U. S. A. 97, 5768–72 (2000).

Heffeter, P. et al. Intrinsic and acquired forms of resistance against the anticancer ruthenium compound KP1019 [indazolium trans-[tetrachlorobis(1H-indazole)ruthenate (III)] (FFC14A). J. Pharmacol. Exp. Ther. 312, 281–9 (2005).

Hendrickson, D. G. et al. Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol. 7, e1000238 (2009).

Hermeking, H. MicroRNAs in the p53 network: micromanagement of tumour suppression. Nat. Rev. Cancer 12, 613–26 (2012).

Higashi, H. et al. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science 295, 683–6 (2002).

Hine, C. & Mitchell, J. R. Calorie restriction and methionine restriction in control of endogenous hydrogen sulfide production by the transsulfuration pathway. Exp. Gerontol. 68, 26–32 (2015).

Hishiki, T. et al. Carbon monoxide: impact on remethylation/transsulfuration metabolism and its pathophysiologic implications. J. Mol. Med. (Berl). 90, 245–54 (2012).

Ho, L. & Crabtree, G. R. Chromatin remodelling during development. Nature 463, 474–84 (2010).

Höck, J. & Meister, G. The Argonaute protein family. Genome Biol. 9, 210 (2008).

Hofstetter, G. et al. Alternative splicing of p53 and p73: the novel p53 splice variant p53delta is an independent prognostic marker in ovarian cancer. Oncogene 29, 1997–2004 (2010).

Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. p53 mutations in human cancers. Science 253, 49–53 (1991).

Honda, R., Tanaka, H. & Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett. 420, 25–27 (1997).

Hong, B., van den Heuvel, A. P. J., Prabhu, V. V, Zhang, S. & El-Deiry, W. S. Targeting tumor suppressor p53 for cancer therapy: strategies, challenges and opportunities. Curr. Drug Targets 15, 80–9 (2014).

Hosoda, M. et al. UFD2a mediates the proteasomal turnover of p73 without promoting p73 ubiquitination. Oncogene 24, 7156–69 (2005).

Howell, S. B., Safaei, R., Larson, C. A. & Sailor, M. J. Copper transporters and the cellular pharmacology of the platinum-containing cancer drugs. Mol. Pharmacol. 77, 887–94 (2010).

Hoyo, C. et al. Body mass index in relation to oesophageal and oesophagogastric junction adenocarcinomas: a pooled analysis from the International BEACON Consortium. Int. J. Epidemiol. 41, 1706–18 (2012).

Hu, B. et al. Gastric cancer: Classification, histology and application of molecular pathology. J. Gastrointest. Oncol. 3, 251–61 (2012).

Huang, D. et al. Cisplatin resistance in gastric cancer cells is associated with HER2 upregulation-induced epithelial-mesenchymal transition. Sci. Rep. 6, 20502 (2016).

Hubbert, C. et al. HDAC6 is a microtubule-associated deacetylase. Nature 417, 455–8 (2002).

Huntzinger, E. & Izaurralde, E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. 12, 99–110 (2011).

Hupp, T. R. et al. Small peptides activate the latent sequence-specific DNA binding function of p53. Cell 83, 237–245 (1995).

Hutt, D. M., Roth, D. M., Vignaud, H., Cullin, C. & Bouchecareilh, M. The histone deacetylase inhibitor, Vorinostat, represses hypoxia inducible factor 1 alpha expression through translational inhibition. PLoS One 9, e106224 (2014).

Irwin, M. et al. Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature 407, 645–8 (2000).

Irwin, M. S. et al. Chemosensitivity linked to p73 function. Cancer Cell 3, 403–410 (2003).

Ishiguro, H., Kimura, M. & Takeyama, H. Role of microRNAs in gastric cancer. World J. Gastroenterol. 20, 5694–9 (2014).

Ito, A. et al. p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J. 20, 1331–40 (2001).

Ito, A. et al. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. EMBO J. 21, 6236–45 (2002).

Jamieson, E. R. & Lippard, S. J. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. Chem. Rev. 99, 2467–98 (1999).

Janowski, B. A. et al. Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. Nat. Struct. Mol. Biol. 13, 787–92 (2006).

Jansson, M. D. & Lund, A. H. MicroRNA and cancer. Mol. Oncol. 6, 590–610 (2012).

Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma - 2nd English Edition -. Gastric Cancer 1, 10–24 (1998).

Jenuwein, T. et al. Translating the histone code. Science 293, 1074–80 (2001).

Ji, Q. et al. Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres. BMC Cancer 8, 266 (2008).

Ji, Q. et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. PLoS One 4, e6816 (2009).

Jiang, C. & Pugh, B. F. Nucleosome positioning and gene regulation: advances through genomics. Nat. Rev. Genet. 10, 161–72 (2009).

Johnson, S. M. et al. RAS is regulated by the let-7 microRNA family. Cell 120, 635–47 (2005).

Jones, P. A. & Baylin, S. B. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. 3, 415–28 (2002).

Jones, P. A. & Martienssen, R. A blueprint for a Human Epigenome Project: the AACR Human Epigenome Workshop. Cancer Res. 65, 11241–6 (2005).

Juan, L. J. et al. Histone deacetylases specifically down-regulate p53-dependent gene activation. J. Biol. Chem. 275, 20436–43 (2000).

Jungwirth, U. et al. Anticancer activity of metal complexes: involvement of redox processes. Antioxid. Redox Signal. 15, 1085–127 (2011).

Kaghad, M. et al. Monoallelically Expressed Gene Related to p53 at 1p36, a Region Frequently Deleted in Neuroblastoma and Other Human Cancers. Cell 90, 809–819 (1997).

Kaneda, A., Matsusaka, K., Aburatani, H. & Fukayama, M. Epstein-Barr virus infection as an epigenetic driver of tumorigenesis. Cancer Res. 72, 3445–50 (2012).

Kang, Z.-H. et al. Histone deacetylase HDAC4 promotes gastric cancer SGC-7901 cells progression via p21 repression. PLoS One 9, e98894 (2014).

Kanwal, R. & Gupta, S. Epigenetic modifications in cancer. Clin. Genet. 81, 303–11 (2012).

Kao, H. Y., Downes, M., Ordentlich, P. & Evans, R. M. Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev. 14, 55–66 (2000).

Karimi, P., Islami, F., Anandasabapathy, S., Freedman, N. D. & Kamangar, F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol. Biomarkers Prev. 23, 700–13 (2014).

Kasinski, A. L. & Slack, F. J. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. Nat. Rev. Cancer 11, 849–64 (2011).

Katayama, H. et al. Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. Nat. Genet. 36, 55–62 (2004).

Kato, H., Tamamizu-Kato, S. & Shibasaki, F. Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J. Biol. Chem. 279, 41966–74 (2004).

Kawai, H., Li, H., Avraham, S., Jiang, S. & Avraham, H. K. Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor alpha. Int. J. cancer 107, 353–8 (2003).

Kelley, J. R. et al. Gastric cancer epidemiology and risk factors. J. Clin. Epidemiol. 56, 1–9 (2003).

Kelly, W. K. & Marks, P. A. Drug insight: Histone deacetylase inhibitors--development of the new targeted anticancer agent suberoylanilide hydroxamic acid. Nat. Clin. Pract. Oncol. 2, 150–7 (2005).

Kelman A.D., Clarke M.J., Edmonds S.D., Peresie H.J. Biological activity of ruthenium purine complexes J. Clin. Hematol. Oncol., 7, 274–288 (1977)

Khvorova, A. et al. Functional siRNAs and miRNAs exhibit strand bias. Cell 115, 209–16 (2003).

Kim, C. H. et al. miRNA signature associated with outcome of gastric cancer patients following chemotherapy. BMC Med. Genomics 4, 79 (2011).

Kim, D. H., Villeneuve, L. M., Morris, K. V & Rossi, J. J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. Nat. Struct. Mol. Biol. 13, 793–7 (2006).

Kim, D. H., Kim, M. & Kwon, H. J. Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. J. Biochem. Mol. Biol. 36, 110–9 (2003).

Klajner, M. et al. Subcellular localization and transport kinetics of ruthenium organometallic anticancer compounds in living cells: a dose-dependent role for amino acid and iron transporters. Inorg. Chem. 53, 5150–8 (2014).

Knutson, S. K. et al. Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. EMBO J. 27, 1017–28 (2008).

Köberle, B., Tomicic, M. T., Usanova, S. & Kaina, B. Cisplatin resistance: Preclinical findings and clinical implications. Biochim. Biophys. Acta - Rev. Cancer 1806, 172–182 (2010).

Kohno, K. et al. Mitochondrial Transcription Factor A and Mitochondrial Genome as Molecular Targets for Cisplatin-Based Cancer Chemotherapy. Int. J. Mol. Sci. 16, 19836–50 (2015).

Kornberg, R. D. Chromatin structure: a repeating unit of histones and DNA. Science 184, 868–71 (1974).

Kornberg, R. D. & Lorch, Y. Interplay between chromatin structure and transcription. Curr. Opin. Cell Biol. 7, 371–375 (1995).

Kouzarides, T. et al. Chromatin modifications and their function. Cell 128, 693–705 (2007).

Kovacs, J. J. et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. Mol. Cell 18, 601–7 (2005).

Kozomara, A. & Griffiths-Jones, S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42, D68–73 (2014).

Krek, A. et al. Combinatorial microRNA target predictions. Nat. Genet. 37, 495–500 (2005).

Krusche, C. A. et al. Histone deacetylase-1 and -3 protein expression in human breast cancer: a tissue microarray analysis. Breast Cancer Res. Treat. 90, 15–23 (2005).

Krützfeldt, J. et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature 438, 685–9 (2005).

Kumar, R. K. et al. Gastric Carcinoma: A Review on Epidemiology, Current Surgical and Chemotherapeutic Options. doi:10.5772/45896

Kurokawa, Y. et al. Multicenter large-scale study of prognostic impact of HER2 expression in patients with resectable gastric cancer. Gastric Cancer 18, 691–7 (2015).

Kwon, H. J., Kim, M. S., Kim, M. J., Nakajima, H. & Kim, K.-W. Histone deacetylase inhibitor FK228

Table indicating fold change in miRNAs' expression obtained during the sequencing.

inhibits tumor angiogenesis. Int. J. cancer 97, 290–6 (2002).

La Thangue, N. B. The yin and yang of E2F-1: balancing life and death. Nat. Cell Biol. 5, 587–9 (2003).

Lai, G. M., Ozols, R. F., Young, R. C. & Hamilton, T. C. Effect of glutathione on DNA repair in cisplatin-resistant human ovarian cancer cell lines. J. Natl. Cancer Inst. 81, 535–9 (1989).

Lapi, E. et al. PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop. Mol. Cell 32, 803–14 (2008).

LAUREN, P. THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. Acta Pathol. Microbiol. Scand. 64, 31–49 (1965).

Le, M. T. N. et al. Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. PLoS Genet. 7, e1002242 (2011).

Le, M. T. N. et al. MicroRNA-125b is a novel negative regulator of p53. Genes Dev. 23, 862–76 (2009).

Lee, J., Bass, A. J. & Ajani, J. A. Gastric Adenocarcinoma: An Update on Genomics, Immune System Modulations, and Targeted Therapy. Am. Soc. Clin. Oncol. Educ. Book 35, 104–11 (2016).

Lee, J.-Y. et al. HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-

Expression of *Let-7e and miR-183* in AGS cell lines transfected 48h with miR-183 or Let-7e specific mimics or antimiRs. *Let-7e and miR-183* levels were assayed by RT-qPCR. Bars are means of fold induction versus the control (Ctl). *, $p < 0,001$ (n=3), compared with the control mimic. No statistical significant difference when compared with the control antimiR as calculated by one-way ANOVA test.

control autophagy. EMBO J. 29, 969–80 (2010).

Lee, R. C. et al. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843–854 (1991).

Lee, Y. et al. The nuclear RNase III Drosha initiates microRNA processing. Nature 425, 415–9 (2003).

Lee, Y. et al. The role of PACT in the RNA silencing pathway. EMBO J. 25, 522–32 (2006).

Lee, Y. et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 23, 4051–60 (2004).

Leidinger, P. et al. High-throughput miRNA profiling of human melanoma blood samples. BMC Cancer 10, 262 (2010).

Lemon, D. D. et al. Promiscuous actions of small molecule inhibitors of the protein kinase D-class IIa HDAC axis in striated muscle. FEBS Lett. 589, 1080–8 (2015).

Leng, R. P. et al. Pirh2, a p53-Induced Ubiquitin-Protein Ligase, Promotes p53 Degradation. Cell 112, 779–791 (2003).

Lerner, A. G. et al. IRE1α Induces Thioredoxin-Interacting Protein to Activate the NLRP3 Inflammasome and Promote Programmed Cell Death under Irremediable ER Stress. Cell Metab. 16, 250–264 (2012).

Leung, A. K. L. et al. MicroRNA functions in stress responses. Mol. Cell 40, 205–15 (2010).

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G. & Morgan, D. R. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. J. Med. Microbiol. 26, 93–9 (1988).

Li, D., Marchenko, N. D. & Moll, U. M. SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. Cell Death Differ. 18, 1904–13 (2011).

Li, J., Lin, Q., Wang, W., Wade, P. & Wong, J. Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. Genes Dev. 16, 687–92 (2002).

Li, Z. & Zhu, W.-G. Targeting histone deacetylases for cancer therapy: from molecular mechanisms to clinical implications. Int. J. Biol. Sci. 10, 757–70 (2014).

Liao, Y. et al. Selection of agents for prevention of cisplatin-induced hepatotoxicity. Pharmacol. Res. 57, 125–131 (2008).

Librizzi, M. et al. Cytotoxic Effects of Jay Amin Hydroxamic Acid (JAHA), a Ferrocene-Based Class I Histone Deacetylase Inhibitor, on Triple-Negative MDA-MB231 Breast Cancer Cells. Chem. Res. Toxicol. 25, 2608–2616 (2012).

Liedtke, C., Groger, N., Manns, M. P. & Trautwein, C. The human caspase-8 promoter sustains basal activity through SP1 and ETS-like transcription factors and can be up-regulated by a p53-dependent mechanism. J. Biol. Chem. 278, 27593–604 (2003).

Lim, L. P. et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433, 769–73 (2005).

Lin, R. J., Sternsdorf, T., Tini, M. & Evans, R. M. Transcriptional regulation in acute promyelocytic leukemia. Oncogene 20, 7204–15 (2001).

Lin, X., Zhao, Y., Song, W.-M. & Zhang, B. Molecular classification and prediction in gastric cancer. Comput. Struct. Biotechnol. J. 13, 448–58 (2015a).

Lin, Y., Wu, Z., Guo, W. & Li, J. Gene mutations in gastric cancer: a review of recent next-generation sequencing studies. Tumour Biol. 36, 7385–94 (2015b).

Lin, Y.-L. et al. p63 and p73 transcriptionally regulate genes involved in DNA repair. PLoS Genet. 5, e1000680 (2009).

Lin, Y. et al. Functional dissection of lysine deacetylases reveals that HDAC1 and p300 regulate AMPK. Nature 482, 251–5 (2012).

Lindsey, J. C., Anderton, J. A., Lusher, M. E. & Clifford, S. C. Epigenetic events in medulloblastoma development. Neurosurg. Focus 19, E10 (2005).

Liu, C. & Russell, R. M. Nutrition and gastric cancer risk: an update. Nutr. Rev. 66, 237–49 (2008).

Liu, T. et al. Histone deacetylase inhibitors: Multifunctional anticancer agents. Cancer Treat. Rev. 32, 157–165 (2006).

Lodrini, M. et al. MYCN and HDAC2 cooperate to repress miR-183 signaling in neuroblastoma. Nucleic Acids Res. 41, 6018–33 (2013).

Logotheti, S., Pavlopoulou, A., Galtsidis, S., Vojtesek, B. & Zoumpourlis, V. Functions, divergence and clinical value of TAp73 isoforms in cancer. Cancer Metastasis Rev. 32, 511–34 (2013).

Lu, J. et al. MicroRNA expression profiles classify human cancers. Nature 435, 834–8 (2005).

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. Nuclear export of microRNA precursors. Science 303, 95–8 (2004).

Luo, J., Su, F., Chen, D., Shiloh, A. & Gu, W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature 408, 377–81 (2000).

Luo, R. X. et al. Rb Interacts with Histone Deacetylase to Repress Transcription. Cell 92, 463–473 (1998).

Lyle, R. et al. The imprinted antisense RNA at the Igf2r locus overlaps but does not imprint Mas1. Nat. Genet. 25, 19–21 (2000).

Ma, J., Shen, H., Kapesa, L. & Zeng, S. Lauren classification and individualized chemotherapy in gastric cancer. Oncol. Lett. 11, 2959–2964 (2016).

Macfarlane, L.-A. & Murphy, P. R. MicroRNA: Biogenesis, Function and Role in Cancer. Curr. Genomics 11, 537–61 (2010).

Maison, C. et al. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat. Genet. 30, 329–34 (2002).

Majewski, I. J. et al. An α-E-catenin (CTNNA1) mutation in hereditary diffuse gastric cancer. J. Pathol. 229, 621–9 (2013).

Malhi, H. MICRORNAs IN ER STRESS: DIVERGENT ROLES IN CELL FATE DECISIONS. Curr. Pathobiol. Rep. 2, 117–122 (2014).

Mangiulli, M. et al. Identification and functional characterization of two new transcriptional variants of the human p63 gene. Nucleic Acids Res. 37, 6092–104 (2009).

Mantovani, F. et al. Pin1 Links the Activities of c-Abl and p300 in Regulating p73 Function. Mol. Cell 14, 625–636 (2004).

Marabese, M. et al. p73: A chiaroscuro gene in cancer. Eur. J. Cancer 43, 1361–1372 (2007).

Marcel, V. et al. Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. Cell Death Differ. 18, 1815–24 (2011).

Marchion, D. & Münster, P. Development of histone deacetylase inhibitors for cancer treatment. Expert Rev. Anticancer Ther. 7, 583–98 (2007).

Marek, L. et al. Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells. J. Med. Chem. 56, 427–36 (2013).

Marshall, B. & Warren, J. R. UNIDENTIFIED CURVED BACILLI IN THE STOMACH OF PATIENTS WITH GASTRITIS AND PEPTIC ULCERATION. Lancet 323, 1311–1315 (1984).

Martin, A. & Scharff, M. D. AID and mismatch repair in antibody diversification. Nat. Rev. Immunol. 2, 605 (2002).

Marullo, R. et al. Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS One 8, e81162 (2013).

Maurel, M. & Chevet, E. Endoplasmic reticulum stress signaling: the microRNA connection. Am. J. Physiol. Cell Physiol. 304, C1117–26 (2013).

Mavrich, T. N. et al. A barrier nucleosome model for statistical positioning of nucleosomes throughout the yeast genome. Genome Res. 18, 1073–83 (2008).

McKinsey, T. A., Zhang, C. L., Lu, J. & Olson, E. N. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 408, 106–11 (2000).

McKinsey, T. A., Zhang, C. L. & Olson, E. N. Control of muscle development by dueling HATs and HDACs. Curr. Opin. Genet. Dev. 11, 497–504 (2001).

McWhinney, S. R., Goldberg, R. M. & McLeod, H. L. Platinum neurotoxicity pharmacogenetics. Mol. Cancer Ther. 8, 10–6 (2009).

Medina, V. et al. Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. Cancer Res. 57, 3697–707 (1997).

Meggers, E. Exploring biologically relevant chemical space with metal complexes. Curr. Opin. Chem. Biol. 11, 287–292 (2007).

Melo, S. A. et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. Nat. Genet. 41, 365–70 (2009).

Melo, S. A. et al. A Genetic Defect in Exportin-5 Traps Precursor MicroRNAs in the Nucleus of Cancer Cells. Cancer Cell 18, 303–315 (2010).

Meng, F. et al. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133, 647–58 (2007).

Meng, X. et al. A ruthenium-containing organometallic compound reduces tumor growth through induction of the endoplasmic reticulum stress gene CHOP. Cancer Res. 69, 5458–66 (2009).

Messori, L., Kratz, F. & Alessio, E. The Interaction of the Antitumor Complexes Na[trans-RuCl(4)(DMSO)(Im)] and Na[trans-RuCl(4)(DMSO)(Ind)] With Apotransferrin: a Spectroscopic Study. Met. Based. Drugs 3, 1–9 (1996).

Meyerhardt, J. A. & Mayer, R. J. Systemic Therapy for Colorectal Cancer. http://dx.doi.org/10.1056/NEJMra040958 (2009).

Michael, A. et al. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis. 16, 34–8 (2010).

Mihmanli, M., Ilhan, E., Idiz, U. O., Alemdar, A. & Demir, U. Recent developments and innovations in gastric cancer. World J. Gastroenterol. 22, 4307–20 (2016).

Miller, K. M. et al. Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining. Nat. Struct. Mol. Biol. 17, 1144–51 (2010).

Mills, A. A. et al. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398, 708–13 (1999).

Minty, A., Dumont, X., Kaghad, M. & Caput, D. Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. J. Biol. Chem. 275, 36316–23 (2000).

Minucci, S. & Pelicci, P. G. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat. Rev. Cancer 6, 38–51 (2006).

Mirza, A., Pritchard, S. & Welch, I. The postoperative component of MAGIC chemotherapy is associated with improved prognosis following surgical resection in gastric and gastrooesophageal junction adenocarcinomas. Int. J. Surg. Oncol. 2013, 781742 (2013).

Mistry, R. K. et al. Transcriptional Regulation of Cystathionine-γ-Lyase in Endothelial Cells by NADPH Oxidase 4-Dependent Signaling. J. Biol. Chem. 291, 1774–88 (2016).

Mladenov, E. & Iliakis, G. in DNA Repair - On the Pathways to Fixing DNA Damage and Errors (InTech, 2011). doi:10.5772/24572

Moll, U. M., Erster, S. & Zaika, A. p53, p63 and p73 – solos, alliances and feuds among family members. Biochim. Biophys. Acta - Rev. Cancer 1552, 47–59 (2001).

Moncharmont, C. et al. Cisplatine ou carboplatine, telle est la question. Bull. Cancer 98, 164–175 (2011).

Montagnani, F., Turrisi, G., Marinozzi, C., Aliberti, C. & Fiorentini, G. Effectiveness and safety of oxaliplatin compared to cisplatin for advanced, unresectable gastric cancer: a systematic review and meta-analysis. Gastric Cancer 14, 50–5 (2011).

Mori, Y. et al. The impact of microsatellite instability on the molecular phenotype of colorectal tumors. Cancer Res. 63, 4577–82 (2003).

Mosharov, E., Cranford, M. R. & Banerjee, R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. Biochemistry 39, 13005–11 (2000).

Motoyama, K. et al. Clinical significance of high mobility group A2 in human gastric cancer and its relationship to let-7 microRNA family. Clin. Cancer Res. 14, 2334–40 (2008).

Mottet, D. et al. HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. Oncogene 28, 243–56 (2009).

Mudhasani, R. et al. Loss of miRNA biogenesis induces p19Arf-p53 signaling and senescence in primary cells. J. Cell Biol. 181, 1055–63 (2008).

Müller, M. et al. TAp73/Delta Np73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. Cell Death Differ. 12, 1564–77 (2005).

Munarriz, E. et al. PIAS-1 is a checkpoint regulator which affects exit from G1 and G2 by sumoylation of p73. Mol. Cell. Biol. 24, 10593–610 (2004).

Murphy, M. et al. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev. 13, 2490–501 (1999).

Murray-Zmijewski, F., Lane, D. P. & Bourdon, J.-C. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. Cell Death Differ. 13, 962–72 (2006).

Mutze, K. et al. Histone deacetylase (HDAC) 1 and 2 expression and chemotherapy in gastric cancer. Ann. Surg. Oncol. 17, 3336–43 (2010).

Nalls, D., Tang, S.-N., Rodova, M., Srivastava, R. K. & Shankar, S. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. PLoS One 6, e24099 (2011).

Nan, X. et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393, 386–9 (1998).

Nishida, N. et al. MicroRNA miR-125b is a prognostic marker in human colorectal cancer. Int. J. Oncol. 38, 1437–43 (2011).

Nomura A. Stomach cancer. In Schottenfeld D, Fraumeni JF: Cancer Epidemiology and Prevention, 2nd edition. New York, NY: Oxford University Press 707–724.1, (1996).

Noonan, E. J. et al. miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28, 1714–24 (2009).

Nozell, S. et al. Characterization of p73 functional domains necessary for transactivation and growth suppression. Oncogene 22, 4333–47 (2003).

Nursal and al., Epstein-Barr Virus and DNA Methylation in Gastric Cancer. doi:10.5152/imj. 2016.28482 (2016).

Nurse, P. et al. A Long Twentieth Century of the Cell Cycle and Beyond. Cell 100, 71–78 (2000).

Oda, S., Zhao, Y. & Maehara, Y. Microsatellite instability in gastrointestinal tract cancers: a brief update. Surg. Today 35, 1005–15 (2005).

O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V & Mendell, J. T. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839–43 (2005).

Oehme, I. et al. Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin. Cancer Res. 15, 91–9 (2009).

Oehme, I. et al. Histone deacetylase 10 promotes autophagy-mediated cell survival. Proc. Natl. Acad. Sci. U. S. A. 110, E2592–601 (2013).

Oh, J. D., Karam, S. M. & Gordon, J. I. Intracellular Helicobacter pylori in gastric epithelial progenitors. Proc. Natl. Acad. Sci. U. S. A. 102, 5186–91 (2005).

Oh, S. T. et al. A naturally derived gastric cancer cell line shows latency I Epstein–Barr virus infection closely resembling EBV-associated gastric cancer. Virology 320, 330–336 (2004).

Olena, A. F. & Patton, J. G. Genomic organization of microRNAs. J. Cell. Physiol. 222, 540–5 (2010).

Oliveira, C. et al. Familial gastric cancer: genetic susceptibility, pathology, and implications for management. Lancet. Oncol. 16, e60–70 (2015).

Oliveira, C. et al. Germline CDH1 deletions in hereditary diffuse gastric cancer families. Hum. Mol. Genet. 18, 1545–55 (2009).

Ostrakhovitch, E. A., Akakura, S., Sanokawa-Akakura, R., Goodwin, S. & Tabibzadeh, S. Dedifferentiation of cancer cells following recovery from a potentially lethal damage is mediated by H2S–Nampt. Exp. Cell Res. 330, 135–150 (2015).

Ouchi, K. et al. Therapeutic significance of palliative operations for gastric cancer for survival and quality of life. J. Surg. Oncol. 69, 41–4 (1998).

Oyadomari, S. & Mori, M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 11, 381–9 (2004).

Pacelli, F. et al. Proximal compared with distal gastric cancer: multivariate analysis of prognostic factors. Am. Surg. 67, 697–703 (2001).

Pan, X., Wang, Z.-X. & Wang, R. MicroRNA-21: a novel therapeutic target in human cancer. Cancer Biol. Ther. 10, 1224–32 (2010).

Paoletti X, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, et al. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. JAMA, 303, 1729–1737 (2010).

Park, M.-J. et al. PTEN suppresses hyaluronic acid-induced matrix metalloproteinase-9 expression in U87MG glioblastoma cells through focal adhesion kinase dephosphorylation. Cancer Res. 62, 6318–22 (2002).

Parra, M. Class IIa HDACs - new insights into their functions in physiology and pathology. FEBS J. 282, 1736–44 (2015).

Patel, M. & Hu, B. H. MicroRNAs in inner ear biology and pathogenesis. Hear. Res. 287, 6–14 (2012).

Patel, S. et al. Molecular interactions of ASPP1 and ASPP2 with the p53 protein family and the apoptotic promoters PUMA and Bax. Nucleic Acids Res. 36, 5139–51 (2008).

Perron, M. P. & Provost, P. Protein interactions and complexes in human microRNA biogenesis and function. Front. Biosci. 13, 2537–47 (2008).

Pichiorri, F. et al. Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. Cancer Cell 18, 367–81 (2010).

Pietrantonio, F. et al. A review on biomarkers for prediction of treatment outcome in gastric cancer. Anticancer Res. 33, 1257–66 (2013).

Pillai, R. S., Artus, C. G. & Filipowicz, W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA 10, 1518–25 (2004).

Pinheiro, H. et al. Allele-specific CDH1 downregulation and hereditary diffuse gastric cancer. Hum. Mol. Genet. 19, 943–52 (2010).

Pizarro, A. M. & Sadler, P. J. Unusual DNA binding modes for metal anticancer complexes. Biochimie 91, 1198–1211 (2009).

Portela, A. & Esteller, M. Epigenetic modifications and human disease. Nat. Biotechnol. 28, 1057–68 (2010).

Qian, D. Z. et al. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. Cancer Res. 66, 8814–21 (2006).

Qiu, M. et al. Clinicopathological characteristics and prognostic analysis of Lauren classification in gastric adenocarcinoma in China. J. Transl. Med. 11, 58 (2013).

Rabik, C. A. et al. Role of copper transporters in resistance to platinating agents. Cancer Chemother. Pharmacol. 64, 133–42 (2009).

Racek, T., Mise, N., Li, Z., Stoll, A. & Pützer, B. M. C-terminal p73 isoforms repress transcriptional activity of the human telomerase reverse transcriptase (hTERT) promoter. J. Biol. Chem. 280, 40402–5 (2005).

Rademaker-Lakhai, J. M., van den Bongard, D., Pluim, D., Beijnen, J. H. & Schellens, J. H. M. A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. Clin. Cancer Res. 10, 3717–27 (2004).

Rajewsky, N. microRNA target predictions in animals. Nat. Genet. 38 Suppl, S8–13 (2006).

Raver-Shapira, N. et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol. Cell 26, 731–43 (2007).

Ringrose, L. & Paro, R. Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development 134, 223–32 (2007).

Rivetti di Val Cervo, P. et al. p73, miR106b, miR34a, and Itch in chronic lymphocytic leukemia. Blood 113, 6498–9; author reply 6499–500 (2009).

Rivlin, N., Brosh, R., Oren, M. & Rotter, V. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer 2, 466–74 (2011).

Robb, G. B. et al. RNA helicase A interacts with RISC in human cells and functions in RISC loading. Mol. Cell 26, 523–37 (2007).

Roberts, C. W. M. & Orkin, S. H. The SWI/SNF complex--chromatin and cancer. Nat. Rev. Cancer 4, 133–42 (2004).

Robertson, K. D. DNA methylation and human disease. Nat. Rev. Genet. 6, 597–610 (2005).

Robinson, R. A. et al. Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73. Structure 16, 259–68 (2008).

Röcken, C. et al. Validating the prognostic and discriminating value of the TNM-classification for gastric cancer - a critical appraisal. Eur. J. Cancer 51, 577–86 (2015).

Roder, D. M. The epidemiology of gastric cancer. Gastric Cancer 5, 5–11 (2002).

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 14, 1902–10 (2004).

Rokavec, M., Li, H., Jiang, L. & Hermeking, H. The p53/microRNA connection in gastrointestinal cancer. Clin. Exp. Gastroenterol. 7, 395–413 (2014).

Ropero, S. et al. The role of histone deacetylases (HDACs) in human cancer. Mol. Oncol. 1, 19–25 (2007).

Rosell, R. et al. Nucleotide excision repair pathways involved in Cisplatin resistance in non-small-cell lung cancer. Cancer Control 10, 297–305

Rosenbluth, J. M., Mays, D. J., Pino, M. F., Tang, L. J. & Pietenpol, J. A. A gene signature-based approach identifies mTOR as a regulator of p73. Mol. Cell. Biol. 28, 5951–64 (2008).

Rossi, J. J. Mammalian Dicer finds a partner. EMBO Rep. 6, 927–9 (2005a).

Rossi M, Inoue S, Walewska R, Knight RA, Dyer MJ, Cohen GM, Melino G. Caspase cleavage of Itch in chronic lymphocytic leukemia cells. Biochem Biophys Res Commun 379, 659–664, (2009).

Rossi, M. et al. The ubiquitin-protein ligase Itch regulates p73 stability. EMBO J. 24, 836–48 (2005b).

Roukos, D. H., Agnantis, N. J., Fatouros, M. & Kappas, A. M. MINI-REVIEW Gastric Cancer: Introduction, Pathology, Epidemiology. GBC 1, (2002a).

Roukos, D. H. & Kappas, A. M. Targeting the optimal extent of lymph node dissection for gastric cancer. J. Surg. Oncol. 81, 59–62; discussion 62 (2002b).

Rouleau, E. et al. First large rearrangement in the MUTYH gene and attenuated familial adenomatous polyposis syndrome. Clin. Genet. 80, 301–3 (2011).

Rufini, A. et al. p73 in Cancer. Genes Cancer 2, 491–502 (2011).

Rugge, M. et al. Gastric dysplasia: the Padova international classification. Am. J. Surg. Pathol. 24, 167–76 (2000).

Rusek, A. M. et al. MicroRNA modulators of epigenetic regulation, the tumor microenvironment and the immune system in lung cancer. Mol. Cancer 14, 34 (2015).

Ryabov, A. D., Sukharev, V. S., Alexandrova, L., Le Lagadec, R. & Pfeffer, M. New synthesis and new bio-application of cyclometalated ruthenium(II) complexes for fast mediated electron transfer with peroxidase and glucose oxidase. Inorg. Chem. 40, 6529–32 (2001).

Rybak, A. et al. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat. Cell Biol. 10, 987–93 (2008).

Saad, S. Y., Najjar, T. A. O. & Alashari, M. Role of non-selective adenosine receptor blockade and phosphodiesterase inhibition in cisplatin-induced nephrogonadal toxicity in rats. Clin. Exp. Pharmacol. Physiol. 31, 862–7 (2004).

Sachan, M., Kaur, M., Sachan, M. & Kaur, M. Epigenetic Modifications: Therapeutic Potential in Cancer. Brazilian Arch. Biol. Technol. 58, 526–539 (2015).

Sætrom, P., Snøve, O. & Rossi, J. J. Epigenetics and MicroRNAs. Pediatr. Res. 61, 17R–23R (2007).

Safaei, R. et al. Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. Crit. Rev. Oncol. Hematol. 53, 13–23 (2005).

Safaei, R., Otani, S., Larson, B. J., Rasmussen, M. L. & Howell, S. B. Transport of cisplatin by the copper efflux transporter ATP7B. Mol. Pharmacol. 73, 461–8 (2008).

Sahu, S. K. et al. Epstein–Barr virus nuclear antigen 3C interact with p73: Interplay between a viral oncoprotein and cellular tumor suppressor. Virology 448, 333–343 (2014).

Saito, Y. et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9, 435–443 (2006).

Salzman, D. W., Shubert-Coleman, J. & Furneaux, H. P68 RNA helicase unwinds the human let-7 microRNA precursor duplex and is required for let-7-directed silencing of gene expression. J. Biol. Chem. 282, 32773–9 (2007).

Sampath, D. et al. Specific activation of microRNA106b enables the p73 apoptotic response in chronic lymphocytic leukemia by targeting the ubiquitin ligase Itch for degradation. Blood 113, 3744–53 (2009).

Sanokawa-Akakura, R., Ostrakhovitch, E. A., Akakura, S., Goodwin, S. & Tabibzadeh, S. A H2S-Nampt dependent energetic circuit is critical to survival and cytoprotection from damage in cancer cells. PLoS One 9, e108537 (2014).

Santenard, A. & Torres-Padilla, M.-E. Epigenetic reprogramming in mammalian reproduction: contribution from histone variants. Epigenetics 4, 80–4 (2009).

Sato, F. & Meltzer, S. J. CpG island hypermethylation in progression of esophageal and gastric cancer. Cancer 106, 483–93 (2006a).

Sato, T., Suzuki, M., Sato, Y., Echigo, S. & Rikiishi, H. Sequence-dependent interaction between cisplatin and histone deacetylase inhibitors in human oral squamous cell carcinoma cells. Int. J. Oncol. 28, 1233–41 (2006b).

Sava, G. et al. Dual Action of NAMI-A in inhibition of solid tumor metastasis: selective targeting of metastatic cells and binding to collagen. Clin. Cancer Res. 9, 1898–905 (2003).

Sawa, H. et al. Histone deacetylase inhibitors such as sodium butyrate and trichostatin A induce apoptosis through an increase of the bcl-2-related protein Bad. Brain Tumor Pathol. 18, 109–14 (2001).

Scheeff, E. D., Briggs, J. M. & Howell, S. B. Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin. Mol. Pharmacol. 56, 633–43 (1999).

Schetter, A. J. et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 299, 425–36 (2008).

Schuhmacher, C. et al. Neoadjuvant chemotherapy compared with surgery alone for locally advanced cancer of the stomach and cardia: European Organisation for Research and Treatment of Cancer randomized trial 40954. J. Clin. Oncol. 28, 5210–8 (2010).

Schwamborn, J. C. et al. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell 136, 913–25 (2009).

Schwarz, D. S. et al. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115, 199–208 (2003).

Scolaro, C. et al. In vitro and in vivo evaluation of ruthenium(II)-arene PTA complexes. J. Med. Chem. 48, 4161–71 (2005).

Seetharam, R., Sood, A. & Goel, S. Oxaliplatin: pre-clinical perspectives on the mechanisms of action, response and resistance. Ecancermedicalscience 3, 153 (2009).

Selbach, M. et al. Widespread changes in protein synthesis induced by microRNAs. Nature 455, 58–63 (2008).

Semino-Mora, C. et al. Intracellular and interstitial expression of Helicobacter pylori virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma. J. Infect. Dis. 187, 1165–77 (2003).

Seo, Y. R., Fishel, M. L., Amundson, S., Kelley, M. R. & Smith, M. L. Implication of p53 in base excision DNA repair: in vivo evidence. Oncogene 21, 731–7 (2002).

Serrano, M. et al. Oncogenic ras Provokes Premature Cell Senescence Associated with Accumulation of p53 and p16INK4a. Cell 88, 593–602 (1997).

Sharma, S., Kelly, T. K. & Jones, P. A. Epigenetics in cancer. Carcinogenesis 31, 27–36 (2010).

Shen, D.-W. et al. Elevated expression of TMEM205, a hypothetical membrane protein, is associated with cisplatin resistance. J. Cell. Physiol. 225, 822–8 (2010).

Shen, D.-W., Pouliot, L. M., Hall, M. D. & Gottesman, M. M. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol. Rev. 64, 706–21 (2012).

Shen, J. et al. Enhancement of cisplatin induced apoptosis by suberoylanilide hydroxamic acid in human oral squamous cell carcinoma cell lines. Biochem. Pharmacol. 73, 1901–1909 (2007).

Shen, L. et al. Bevacizumab plus capecitabine and cisplatin in Chinese patients with inoperable locally advanced or metastatic gastric or gastroesophageal junction cancer: randomized, double-blind, phase III study (AVATAR study). Gastric Cancer 18, 168–76 (2015).

Shi, Y. Histone lysine demethylases: emerging roles in development, physiology and disease. Nat. Rev. Genet. 8, 829–33 (2007).

Shiloh, Y. ATM and ATR: networking cellular responses to DNA damage. Curr. Opin. Genet. Dev. 11, 71–77 (2001).

Shimizu, Y., Iwai, S., Hanaoka, F. & Sugasawa, K. Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase. EMBO J. 22, 164–73 (2003).

Shimono, Y. et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138, 592–603 (2009).

Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 22, 7265–79 (2003).

Siurala, M., Varis, K. & Wiljasalo, M. Studies of patients with atrophic gastritis: a 10-15-year follow-up. Scand. J. Gastroenterol. 1, 40–8 (1966).

Sjödahl, K. et al. Smoking and alcohol drinking in relation to risk of gastric cancer: a population-based, prospective cohort study. Int. J. cancer 120, 128–32 (2007).

Smalley, K. S. M. et al. An organometallic protein kinase inhibitor pharmacologically activates p53 and induces apoptosis in human melanoma cells. Cancer Res. 67, 209–17 (2007).

Smith, C. L. & Peterson, C. L. ATP-Dependent Chromatin Remodeling.

Song, B. et al. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. Oncogene 28, 4065–74 (2009).

Spencer, J. et al. Synthesis and Biological Evaluation of JAHAs: Ferrocene-Based Histone Deacetylase Inhibitors. ACS Med. Chem. Lett. 2, 358–362 (2011).

Spreckelmeyer, S., Orvig, C. & Casini, A. Cellular transport mechanisms of cytotoxic metallodrugs: an overview beyond cisplatin. Molecules 19, 15584–610 (2014).

St Clair, S. & Manfredi, J. J. The dual specificity phosphatase Cdc25C is a direct target for transcriptional repression by the tumor suppressor p53. Cell Cycle 5, 709–13 (2006).

Steele, N., Finn, P., Brown, R. & Plumb, J. A. Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo. Br. J. Cancer 100, 758–63 (2009).

Stiewe, T. The p53 family in differentiation and tumorigenesis. Nat. Rev. Cancer 7, 165–8 (2007).

Stiewe, T. et al. Inactivation of retinoblastoma (RB) tumor suppressor by oncogenic isoforms of the p53 family member p73. J. Biol. Chem. 278, 14230–6 (2003).

Stiewe, T., Theseling, C. C. & Pützer, B. M. Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. J. Biol. Chem. 277, 14177–85 (2002).

Stronach, E. A. et al. HDAC4-regulated STAT1 activation mediates platinum resistance in ovarian cancer. Cancer Res. 71, 4412–22 (2011).

Su, X. et al. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature 467, 986–90 (2010).

Sun, L. et al. MiR-200b and miR-15b regulate chemotherapy-induced epithelial-mesenchymal transition in human tongue cancer cells by targeting BMI1. Oncogene 31, 432–45 (2012).

Suzuki, H. I. et al. Modulation of microRNA processing by p53. Nature 460, 529–33 (2009a).

Suzuki, K. & Matsubara, H. Recent advances in p53 research and cancer treatment. J. Biomed. Biotechnol. 2011, 978312 (2011).

Suzuki, M., Endo, M., Shinohara, F., Echigo, S. & Rikiishi, H. Enhancement of cisplatin cytotoxicity by SAHA involves endoplasmic reticulum stress-mediated apoptosis in oral squamous cell carcinoma cells. Cancer Chemother. Pharmacol. 64, 1115–22 (2009b).

Suzuki, M. M. & Bird, A. DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. 9, 465–76 (2008).

Swierczynski, S. et al. Histone deacetylation meets miRNA: epigenetics and post-transcriptional regulation in cancer and chronic diseases. Expert Opin. Biol. Ther. 15, 651–64 (2015).

Taganov, K. D., Boldin, M. P., Chang, K.-J. & Baltimore, D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc. Natl. Acad. Sci. U. S. A. 103, 12481–6 (2006).

Tait, S. W. G. & Green, D. R. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. 11, 621–32 (2010).

Takagi, T. et al. Decreased expression of microRNA-143 and -145 in human gastric cancers. Oncology 77, 12–21 (2009).

Takai, D. & Jones, P. A. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc. Natl. Acad. Sci. U. S. A. 99, 3740–5 (2002).

Takamizawa, J. et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 64, 3753–6 (2004).

Takimoto, R. et al. Augmentation of antitumor effects of p53 gene therapy by combination with HDAC inhibitor. Cancer Biol. Ther. 4, 421–8 (2005).

Tang, X. et al. Glycogen synthase kinase 3 beta inhibits microRNA-183-96-182 cluster via the β-Catenin/TCF/LEF-1 pathway in gastric cancer cells. Nucleic Acids Res. 42, 2988–98 (2014).

Tarasov, V. et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle 6, 1586–93 (2007).

Taylor, D., and Parsonnet, J. Infections of the gastrointestinal tract. In Infections of the gastrointestinal tract. Raven Press. New York, New York, USA, 551–563, (1995).

Tazawa, H., Tsuchiya, N., Izumiya, M. & Nakagama, H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proc. Natl. Acad. Sci. U. S. A. 104, 15472–7 (2007).

TERRY, M., GAUDET, M. & GAMMON, M. The epidemiology of gastric cancer. Semin. Radiat. Oncol. 12, 111–127 (2002).

Terui, T. et al. Induction of PIG3 and NOXA through acetylation of p53 at 320 and 373 lysine residues as a mechanism for apoptotic cell death by histone deacetylase inhibitors. Cancer Res. 63, 8948–54 (2003).

Thornberry, N. A. & Lazebnik, Y. Caspases: enemies within. Science 281, 1312–6 (1998).

Toledo, F. & Wahl, G. M. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat. Rev. Cancer 6, 909–23 (2006).

Tomasini, R. et al. TAp73 regulates the spindle assembly checkpoint by modulating BubR1 activity. Proc. Natl. Acad. Sci. U. S. A. 106, 797–802 (2009).

Tomasini, R. et al. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. Genes Dev. 22, 2677–91 (2008).

Tong, J. J., Liu, J., Bertos, N. R. & Yang, X.-J. Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. Nucleic Acids Res. 30, 1114–23 (2002).

Torre, L. A. et al. Global cancer statistics, 2012. CA. Cancer J. Clin. 65, 87–108 (2015).

Trang, P. et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Mol. Ther. 19, 1116–22 (2011).

Trondl, R. et al. NKP-1339, the first ruthenium-based anticancer drug on the edge to clinical application. Chem. Sci. 5, 2925–2932 (2014).

Tsukamoto, Y. et al. Genome-wide analysis of DNA copy number alterations and gene expression in gastric cancer. J. Pathol. 216, 471–82 (2008).

Tsukamoto, Y. et al. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. Cancer Res. 70, 2339–49 (2010).

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M. & Hatakeyama, M. Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. J. Biol. Chem. 278, 3664–70 (2003).

Urist, M. et al. The linchpin? Pin1 meets p73. Cancer Cell 5, 515–517 (2004b).

Urist, M., Tanaka, T., Poyurovsky, M. V & Prives, C. p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. Genes Dev. 18, 3041–54 (2004a).

Ushijima, T. et al. Focus on gastric cancer. Cancer Cell 5, 121–125 (2004).

Vacca, A. et al. Inhibition of endothelial cell functions and of angiogenesis by the metastasis inhibitor NAMI-A. Br. J. Cancer 86, 993–8 (2002).

Vandermeers, F., Kettmann, R. & Willems, L. Implication des modifications épigénétiques dans les cancers : développement de nouvelles approches thérapeutiques. Biotechnol. Agron. Soc. Environ. (2008)

Vasquez, K. M., Christensen, J., Li, L., Finch, R. A. & Glazer, P. M. Human XPA and RPA DNA repair proteins participate in specific recognition of triplex-induced helical distortions. Proc. Natl. Acad. Sci. U. S. A. 99, 5848–53 (2002).

Vauhkonen, M. et al. Pathology and molecular biology of gastric cancer. Best Pract. Res. Clin. Gastroenterol. 20, 651–674 (2006).

Vega, R. B. et al. Histone Deacetylase 4 Controls Chondrocyte Hypertrophy during Skeletogenesis. Cell 119, 555–566 (2004).

Vernole, P. et al. TAp73alpha binds the kinetochore proteins Bub1 and Bub3 resulting in polyploidy. Cell Cycle 8, 421–9 (2009).

Vidimar, V. et al. Induction of caspase 8 and reactive oxygen species by ruthenium-derived anticancer compounds with improved water solubility and cytotoxicity. Biochem. Pharmacol. 84, 1428–36 (2012).

Villar-Garea, A. & Esteller, M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Curr. Drug Metab. 4, 11–31 (2003).

Viswanathan, S. R., Daley, G. Q. & Gregory, R. I. Selective blockade of microRNA processing by Lin28. Science 320, 97–100 (2008).

Vousden, K. H. et al. Blinded by the Light: The Growing Complexity of p53. Cell 137, 413–31 (2009).

Wade, P. A. Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum. Mol. Genet. 10, 693–8 (2001).

Wang, D. & Lippard, S. J. Cellular processing of platinum anticancer drugs. Nat. Rev. Drug Discov. 4, 307–20 (2005).

Wang, Q.-E., Han, C., Milum, K. & Wani, A. A. Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. Mutat. Res. Mol. Mech. Mutagen. 708, 59–68 (2011).

Wang, Y. & Leung, F. C. C. An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 20, 1170–7 (2004).

Wang, Z., Qin, G. & Zhao, T. C. HDAC4: mechanism of regulation and biological functions. Epigenomics 6, 139–50 (2014).

Watt, P. M., Kumar, R. & Kees, U. R. Promoter demethylation accompanies reactivation of the HOX11 proto-oncogene in leukemia. Genes. Chromosomes Cancer 29, 371–7 (2000).

Wei, J. et al. Pathogenic bacterium Helicobacter pylori alters the expression profile of p53 protein isoforms and p53 response to cellular stresses. Proc. Natl. Acad. Sci. U. S. A. 109, E2543–50 (2012).

Wei, J. et al. Bacterial CagA protein induces degradation of p53 protein in a p14ARF-dependent manner. Gut 64, 1040–8 (2015).

Weiss, M. M. et al. Genomic alterations in primary gastric adenocarcinomas correlate with clinicopathological characteristics and survival. Cell. Oncol. 26, 307–17 (2004).

Welsh, C. et al. Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. Int. J. cancer 110, 352–61 (2004).

Wermann, H. et al. Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance. J. Pathol. 221, 433–42 (2010).

Werner, M., Becker, K. F., Keller, G. & Höfler, H. Gastric adenocarcinoma: pathomorphology and molecular pathology. J. Cancer Res. Clin. Oncol. 127, 207–16 (2001).

Wilhelm, M. T. et al. Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. Genes Dev. 24, 549–60 (2010).

Wilkinson, S. M., Uhl, J. R., Kline, B. C. & Cockerill, F. R. Assessment of invasion frequencies of cultured HEp-2 cells by clinical isolates of Helicobacter pylori using an acridine orange assay. J. Clin. Pathol. 51, 127–33 (1998).

Wilson, A. J. et al. HDAC4 promotes growth of colon cancer cells via repression of p21. Mol. Biol. Cell 19, 4062–75 (2008).

Wiltshaw E. Cisplatin in the treatment of cancer. Platinum Metals Review, 23(3), 90-8, (1979)

Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat. Cell Biol. 11, 228–34 (2009).

Witt, O., Deubzer, H. E., Milde, T. & Oehme, I. HDAC family: What are the cancer relevant targets? Cancer Lett. 277, 8–21 (2009).

Wittekind, C. The development of the TNM classification of gastric cancer. Pathol. Int. 65, 399–403 (2015).

Worthley, D. L. et al. Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): a new autosomal dominant syndrome. Gut 61, 774–9 (2012).

Woźniak, K. & Błasiak, J. Recognition and repair of DNA-cisplatin adducts. Acta Biochim. Pol. 49, 583–96 (2002).

Wu, G. S. et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. 17, 141–3 (1997).

Wu, S. C. & Zhang, Y. Active DNA demethylation: many roads lead to Rome. Nat. Rev. Mol. Cell Biol. 11, 607–20 (2010).

Wutz, A. et al. Imprinted expression of the Igf2r gene depends on an intronic CpG island. Nature 389, 745–9 (1997).

Xia, L. et al. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int. J. cancer 123, 372–9 (2008).

Xiao, B. et al. Detection of miR-106a in gastric carcinoma and its clinical significance. Clin. Chim. Acta 400, 97–102 (2009).

Xiao, J., Lin, H., Luo, X., Luo, X. & Wang, Z. miR-605 joins p53 network to form a p53:miR-605:Mdm2 positive feedback loop in response to stress. EMBO J. 30, 5021 (2011).

Xirodimas, D. P., Chisholm, J., Desterro, J. M. ., Lane, D. P. & Hay, R. T. P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2. FEBS Lett. 528, 207–211 (2002).

Xirodimas, D. P. et al. Mdm2-Mediated NEDD8 Conjugation of p53 Inhibits Its Transcriptional Activity. Cell 118, 83–97 (2004).

Xue, W. et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 445, 656–60 (2007).

Yadav, R. K. et al. Endoplasmic Reticulum Stress and Cancer. J. Cancer Prev. 19, 75–88 (2014).

Yamakuchi, M. et al. P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. Proc. Natl. Acad. Sci. U. S. A. 107, 6334–9 (2010).

Yan, H. et al. Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. EMBO J. 28, 2719–32 (2009).

Yan, W. et al. Histone deacetylase inhibitors suppress mutant p53 transcription via histone deacetylase 8. Oncogene 32, 599–609 (2013).

Yanaihara, N. et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9, 189–198 (2006).

Yang, A. et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404, 99–103 (2000).

Yang, A. et al. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet. 18, 90–95 (2002b).

Yang, A. et al. p63, a p53 Homolog at 3q27–29, Encodes Multiple Products with Transactivating, Death-Inducing, and Dominant-Negative Activities. Mol. Cell 2, 305–316 (1998).

Yang, G., Thompson, M. A., Brandt, S. J. & Hiebert, S. W. Histone deacetylase inhibitors induce the degradation of the t(8;21) fusion oncoprotein. Oncogene 26, 91–101 (2007).

Yang, H. et al. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res. 68, 425–33 (2008).

Yang, W.-M., Tsai, S.-C., Wen, Y.-D., Fejer, G. & Seto, E. Functional domains of histone deacetylase-3. J. Biol. Chem. 277, 9447–54 (2002a).

Yao, Y.-L. & Yang, W.-M. Beyond histone and deacetylase: an overview of cytoplasmic histone deacetylases and their nonhistone substrates. J. Biomed. Biotechnol. 2011, 146493 (2011).

Yekta, S., Shih, I.-H. & Bartel, D. P. MicroRNA-directed cleavage of HOXB8 mRNA. Science 304, 594–6 (2004).

Yoo, C. B. & Jones, P. A. Epigenetic therapy of cancer: past, present and future. Nat. Rev. Drug Discov. 5, 37–50 (2006).

Yoo, C. et al. Phase I and pharmacodynamic study of vorinostat combined with capecitabine and cisplatin as first-line chemotherapy in advanced gastric cancer. Invest. New Drugs 32, 271–8 (2014).

Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47–59 (2008).

Yu, X. et al. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. J. Natl. Cancer Inst. 94, 504–13 (2002).

Yu-Lun L., Kuo-Wang T., Wen-Chang L. miRNAs in Gastric Cancer, Gastric Carcinoma-Molecular Aspects and Current Advances, Prof. Mahmoud Lotfy (Ed.), ISBN: 978-953-307-412-2, (2011).

Yuan, X. et al. Clustered microRNAs' coordination in regulating protein-protein interaction network. BMC Syst. Biol. 3, 65 (2009).

Yuan, Z. M. et al. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature 399, 814–7 (1999).

Zeng, X., Lee, H., Zhang, Q. & Lu, H. p300 does not require its acetylase activity to stimulate p73 function. J. Biol. Chem. 276, 48–52 (2001).

Zeng, Y. & Cullen, B. R. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. Nucleic Acids Res. 32, 4776–85 (2004).

Zhan, Q. et al. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 18, 2892–900 (1999).

Zhang, H. et al. MiR-138 inhibits tumor growth through repression of EZH2 in non-small cell lung cancer. Cell. Physiol. Biochem. 31, 56–65 (2013a).

Zhang, J. & Zhong, Q. Histone deacetylase inhibitors and cell death. Cell. Mol. Life Sci. 71, 3885–901 (2014b).

Zhang, J., Xu, E. & Chen, X. TAp73 protein stability is controlled by histone deacetylase 1 via regulation of Hsp90 chaperone function. J. Biol. Chem. 288, 7727–37 (2013c).

Zhang, L. et al. An Anticancer Role of Hydrogen Sulfide in Human Gastric Cancer Cells. Oxid. Med. Cell. Longev. 2015, 636410 (2015).

Zhang, Q.-H. et al. Meta-analysis of microRNA-183 family expression in human cancer studies comparing cancer tissues with noncancerous tissues. Gene 527, 26–32 (2013b).

Zhang, W. TCGA divides gastric cancer into four molecular subtypes: implications for individualized therapeutics. Chin. J. Cancer 33, 469–70 (2014a).

Zhang, Y. et al. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev. 13, 1924–35 (1999).

Zhang, Z. et al. Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast*. Breast Cancer Res. Treat. 94, 11–6 (2005).

Zhang, Z. et al. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. Lab. Invest. 88, 1358–66 (2008).

Zhao, Y. et al. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). Mol. Cell. Biol. 26, 2782–90 (2006).

Zhou, C. et al. Suberoylanilide hydroxamic acid enhances the antitumor activity of oxaliplatin by reversing the oxaliplatin−induced Src activation in gastric cancer cells. Mol. Med. Rep. 10, 2729–35 (2014).

Zhou, X., Marks, P. A., Rifkind, R. A. & Richon, V. M. Cloning and characterization of a histone deacetylase, HDAC9. Proc. Natl. Acad. Sci. U. S. A. 98, 10572–7 (2001).

Zhou, X., Richon, V. M., Rifkind, R. A. & Marks, P. A. Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5. Proc. Natl. Acad. Sci. U. S. A. 97, 1056–61 (2000).

Zhu, S. et al. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 18, 350–9 (2008).

Zilberman, D., Coleman-Derr, D., Ballinger, T. & Henikoff, S. Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature 456, 125–9 (2008).

Zilfou, J. T. & Lowe, S. W. Tumor suppressive functions of p53. Cold Spring Harb. Perspect. Biol. 1, a001883 (2009).

Zong, Y. et al. Downregulation of Endogenous Hydrogen Sulfide Pathway Is Involved in Mitochondrion-Related Endothelial Cell Apoptosis Induced by High Salt. Oxid. Med. Cell. Longev. 2015, 754670 (2015).

Marie-Elodie SPAETY

Implication des régulations épigénétiques dans la réponse aux chimiothérapies dans le cancer gastrique – Perspectives thérapeutiques

UNIVERSITÉ DE STRASBOURG

Ecole Doctorale
des Sciences
de la Vie
et de la Santé
STRASBOURG

Résumé

Le cancer gastrique (CG) est traité par résection chirurgicale combinée à une chimiothérapie à base de composés de platine. La résistance croissante aux chimiothérapies renforce la nécessité d'identifier des marqueurs moléculaires robustes pour adapter le traitement et développer des thérapies ciblées.

Durant ma thèse, j'ai montré l'importance des voies de l'épigénétique dans le mode d'action de drogues anti-cancéreuses dans le CG. Notamment, j'ai identifié le rôle d'une histone déacétylase, HDAC4, et de plusieurs miRNAs, dont miR-140, dans la réponse au cisplatine. De plus, j'ai démontré que des composés à base de ruthénium ayant des propriétés redox agissent indépendant de l'ADN et de p53 mais affectent certaines régulations épigénétiques. Ceci m'a donc conduit à étudier l'intérêt thérapeutique et les mécanismes sous-jacents d'un traitement combiné associant le cisplatine et des inhibiteurs de HDAC.

L'ensemble de ces résultats permet d'ouvrir de nouvelles perspectives dans la compréhension des mécanismes d'action des drogues anticancéreuses dans le CG et dans l'identification de marqueurs pronostiques ou de thérapie innovante plus adaptée.

Mots clés: cancer gastrique, platine, ruthenium, épigénétique, HDAC, miRNA, p53 family.

Sumary

Gastric cancer (GC) is treated by surgical resection combined with chemotherapy based on platinum compounds. The increase in chemotherapy resistance reinforces the need to identify robust molecular markers to tailor treatment and develop targeted therapies.

During my PhD, I examined the importance of the epigenetic pathways in the mode of action of anticancer drugs in gastric cancer. In particular, I have identified the role of one histone deacetylase, HDAC4, and several miRNAs, including miR-140, in response to cisplatin. Moreover, I have shown that ruthenium compounds having redox properties act independently of DNA and p53 but affect some epigenetic regulations. This then led me to investigate the therapeutic value and the underlying mechanisms of a combined therapy associating cisplatin and HDAC inhibitors.

All these results will open new perspectives in the understanding of the mechanisms of action of anticancer drugs in gastric cancer and in the identification of prognostic markers or more appropriate advanced therapy.

Keywords: Gastric cancer, platinum, ruthenium, epigenetic, HDAC, miRNA, p53 family.