Understanding the Functional Implications of Cisplatin-Induced Epigenetic Alterations in Osteosarcoma Cells

THESIS

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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2018PHXF0003P

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2024

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis titled **Understanding the Functional Implications of Cisplatin-Induced Epigenetic Alterations in Osteosarcoma Cells** submitted by **Ms.Ankita Daiya** ID No **2018PHXF0003P** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

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Acknowledgements

"Human knowledge is never contained in one person. It grows from the relationships we create between each other and the world, and still, it is never complete"

-Paul Kalanithi, When Breath Becomes Air

As I look back over this PhD journey, one that had its own crests and troughs, one thing remained unchanged- the support of people around me. If you have shuffled the pages to read this, you will know how important you have been to me. Here is a small attempt to pay my gratitude to you all who have stood beside me, in every way.

Firstly, I would like to express my sincerest gratitude to my PhD supervisor Prof. Sudeshna Mukherjee, Department of Biological Sciences, BITS-Pilani. I would always be immensely grateful for the faith she showed in me, for making me power through this entire journey. I would also like to thank my co-supervisor Prof. Shibasish Chowdhury, Department of Biological Sciences, BITS-Pilani for providing me the opportunity to work under his supervision. Their guidance, encouragement, and timely inputs have been instrumental in successful completion of this thesis.

I would like to acknowledge my PhD Doctoral Committee Members - Prof. Rajdeep Chowdhury and Prof. Syamantak Majumder for taking the time to read my work critically and offer their valuable advice. Thank you, for also motivating me to look beyond the obvious.

I acknowledge Prof. V. Ramgopal Rao, Vice-Chancellor, BITS Pilani, and Prof. Sudhirkumar Barai, Director, BITS Pilani, Pilani campus for providing me the infrastructure to carry out the research in their premier institute, BITS-Pilani, Pilani Campus. I would also like to thank Prof. Shamik Chakraborty, Associate Dean, AGSRD, Prof. Jitendra Panwar, Former Associate Dean, AGSRD, Prof. P.N. Jha, Convener, Departmental Research Committee, Department of

Biological Sciences, Prof. Rajdeep Chowdhury, Head of the Department, Department of Biological Sciences, and Prof. P.R. Deepa, Former HoD, Department of Biological Sciences Pilani for their timely guidance and support regarding academic formalities throughout the thesis tenure. I also appreciate the support of all the faculty members of the Department of Biological Sciences. Their queries and suggestions in all the departmental presentations came in handy. I would miss the casual 'hanging in there' of Prof. A.K. Das, and the small conversations about my well-being and aspirations with Prof. Pankaj Kumar Sharma. Being part of the department for almost a decade now, I have learned academic and life lessons from this place. From a UPSC aspirant to a researcher, the Department has been instrumental in my overall academic transformation. A crucial segment of the Department is our very own non-teaching staff, especially Mr. Mukesh Ji, for providing me with all the assistance for my experiments. Mr. Soni Ji and Dr. Iti Sharma for helping me navigate the tedious administrative procedures. And Mr. Ajay Ji, Mr. Subhash Ji, Mr. Naresh Ji for all instrument-related queries.

Finally, I would thank CSIR India for providing me with the fellowship. A timely disbursement would have been greatly appreciated though!

The daily nitty-gritty of this rather adventurous journey has been witnessed closely by the following people, and if you see your name here, know that you have been eternally etched in here. To begin with I would like to thank my seniors - Dr. Subhra Dash, Dr. Leena Fageria, Dr. Vidushi Asati, Dr. Sandeep, Dr. Poonam, Dr. Swati Sharma, Dr. Sweta Karnam, Dr. Abhilasha Srivastava, Dr. Harshita Sharma, Dr. Astha Mittal, Dr. Sumukh Thakar for always listening to me and helping me.

I am eternally grateful to my lab mates - Mr. Anirudh Kumar Sahu, Ms. Propanna Bandhyopadhyay, Ms. Subhashree Chatterjee, Mr. Chinmay Nayak, Ms. Simran Khanna, Ms. Lavanya Suresh Iyer, Ms. Anasuya Banerjee, Ms. Priya Gupta, and Mr. Nachiket Dattatray

Garge for their cheerfulness, their motivation whenever things didn't turn up, for being a kind and supportive bunch of people.

One of the most beautiful bonds I had the privilege of forging here was with Dr. Heena Saini. She is always a call away for solving all my problems- professional or personal. Truly grateful for all the mind-stimulating Scientific discussions and chai sessions at Sky. Special mention to Master Agastya Saini for all those video calls and for being my blob of happiness.

The three essential catalysts of my journey - Ms. Ankita Sharma, Ms. Mahima Choudhary, and Dr. Himanshu Sankrityayan- I thank you all for all your patience, valuable advice and fun times that we had. Thank you for making this quest memorable.

I would like to thank Ms. Sonia, Dr. Nidhi Bub, Mr. Shobham, Ms. Niyati, Mr. S. Ramakrishna, Mr. Srinjoy, Ms. Ashima, Mr. Yash, Mr. Chandrakant, Ms. Hansa, Mr. Farhanur Rahman, Ms. Rubi, Mr. Chetan, Mr. Chetan V.N, Ms. Smita, Ms. Swarnima, Ms. Harsha, Mr. Shreyas, Ms. Nilanjana Mani, and Mr. Nizam for creating a very welcoming and supportive community on Campus. I would also like to acknowledge the research scholars of Department of Biological Sciences. I appreciate Master's Thesis students- Ms. Vishakha, Ms. Shivangi, Ms. Sanhita, Ms. Tejaswini, Ms. Shrinidhi, Mr. Santonu, and Ms. Amrutha Shree, who were always geared up to learn and help. I would also like to sincerely appreciate the help of fellow scholars of the Department of Pharmacy- Dr. Ajinath, Mr. Vishwadeep, Ms. Kavya Shree, Dr. Vivek, Ms. Neha.

I would also like to thank my non-PhD friends who have supported me in ways that they knew- listening to my rants after a failed day. You all are precious!

To my parents- I stand here only because you fostered the courage to go out of your way to make my aspirations come true and for always believing in me. I owe it all to you.

To my siblings, Divya and Bhervesh, for bringing a smile to my face every day and pushing me to improve every day.

To my better half, Rohit - you have been the peace to my chaos. I can't thank you enough for your unconditional support and love. I want to take a moment to express my gratitude to our baby bird - Totu. Wherever you are, I know you are happy and are bestowing us with your blessings. I appreciate my in-laws for being supportive during the last leg of this arduous journey.

To sum it up, I am forever grateful to the Almighty for everything.

Lastly, I would like to dedicate this thesis to my maternal grandfather, my Nanu.

Ankita Daiya

Abstract

Cancer is one of the leading causes of death worldwide. In the last few decades, we have seen advancements to improve the survival rates of cancer patients. However, drug resistance still impedes treatment and is one of the major causes of relapse as well. Diverse drug-resistance mechanism can arise, predominantly driven by pre-existing mutations before treatment, but they have been extensively studied. However, currently there is a growing interest in understanding the epigenetic regulation of resistance and how the understanding of it can aid in formulating better treatment options.

In this regard, osteosarcoma (OS), although a rare cancer, is one of the most aggressive cancers and shows considerable resistance to chemotherapy. Herein, our earlier studies delineate key transcriptomic alterations crucial to survival of OS cells post drug (cispatin; CDDP) exposure. However, the epigenomic alterations governing the transcriptomic changes facilitating survival of these tumor cells are still elusive. This could provide fundamental cues to augment the current therapeutic regime for OS. In this regard, based on our transcriptome data, we first segregated the different epigenetic modifiers based on their action, and found that a vast majority of chromatin-modifying enzymes are dysregulated. Thereafter, we took available patient datasets from GDC and GEO portals and correlated the expression pattern of the epigenetic modulators in osteosarcoma. Upon analysis, we found ~1% genes that were common to both patient and in vitro transcriptome data. We performed gene ontology, pathway and key gene analysis for the same and found histone deacetylases and histone methyltransferases to be amongst key genes regulating the network. This followed an *in vitro* validation of the role of epigenetic modifiers in OS cells upon drug exposure. Interestingly, we found an increased level of repressive marks- H3K27me3 and H3K9me3 after CDDP treatment. Consequently, since we obtained significant change in H3K27me3 levels, we further explored the expression of its catalyst – EZH2 and found it to be increasing with drug treatment.

Based on these observations and existing reports of the role of H3K7me3 in cancer progression, we next explored the functional significance of the enrichment of repressive marks and found it to be coupled to a growth arrest of OS cells, marked not only by an increased expression of p21 (cell cycle inhibitor) but also decreased expression of cell division-associated genes like CyclinA2 (CCNA2). Importantly, an enrichment of H3K27me3 was observed at the upstream promoter element of CCNA2 by ChIP-qPCR. Thereafter, we investigated the upstream cause to the induction of repressive chromatin marks upon CDDP exposure. Majorly, genotoxic stressors such as anticancer drugs often accompany increased reactive oxygen species (ROS) levels, and redox homeostasis is fundamentally crucial for cells in a tumour milieu. In our study, an increased cellular ROS was detected; intriguingly, we found that quenching of ROS reversed the chromatin and growth inhibitory signatures. Having established the growth-inhibitory functional implication of chromatin changes, we henceforth explored molecular signalling involved in the same. Interestingly, our sequencing dataset, indicated reduced expression of LATS1- a negative regulator of the master transcriptional regulator Yes Associated Protein (YAP). This served as a key cue. We observed that after CDDP treatment, YAP signalling and transcriptional activity is enhanced. Also, an increased enrichment of repressive H3K27me3 was found at the promoter of LATS1- the negative regulator of YAP. Moreover, YAP upregulation was coupled to ROS, and inhibition of ROS led to decreased YAP activity.

Herein, existing studies report involvement of YAP in mediating histone acetylation, however, role of YAP in mediating histone methylation is underexplored. Interestingly, pharmacological or genetic ablation of YAP attenuated H3K27me3 levels, and YAP was also found to co-localise with EZH2- the enzymatic catalytic mediator of H3K27me3. Additionally, inhibition of YAP/EZH2 or reversion of chromatin repressive signature by HDAC inhibitors (like SAHA) rendered OS cells more susceptible to the parent drug CDDP, thus providing a therapeutic

alternative. Overall, our study demonstrates an interplay of oxidative stress, YAP signalling and epigenetic modifications in modulating OS cell fate post cisplatin exposure, which can be further explored for effective sensitization of OS cells.

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List of abbreviations:

OS: Osteosarcoma

ecDNA: Extrachromosomal DNA

CDDP: Cisplatin

MAP: High Dose Methotrexate, Doxorubicin, Cisplatin combination

ADM: Adriamycin

DHFR: Dihydrofolate reductase

LSD1: Lysine Demethylase 1

HDAC: Histone Deacetylase

HAT: Histone Acetyl Transferase

DNMT: DNA methyl transferase

lncRNA: Long non coding RNA

CML: Chronic Myeloid Leukemia

AML: Acute Myeloid Leukemia

TMZ: Temozolomide

NSCLC: Non-small cell lung carcinoma

DOT1: Disruptor of Telomeric Silencing 1

EZH2: Enhancer of Zeste Homolog 2

KDM6B: Lysine Demethylase 6B

SUV420H2: Suppressor of Variegation 4-20 Homolog 2 (*Drosophila)*

DXR: Doxorubicin

PRC1/2: Polycomb Repressive Complex ½

YAP: Yes activated Protein

PDAC: Pancreatic Ductal Adenocarcinoma

SWI/SNF: Switch/Sucrose Non-Fermentable

HOS: Human Osteosarcoma

MTT: 3-[4,5-dimethylthiazol-2yl]-2,5 diphenyl tetrazolium bromide

ROS: Reactive Oxygen Species

DCFDA: 2'-7'-Dichlorofluorescein diacetate

CCNA2: Cyclin A2

PI: Propidium Iodide

PCNA: Proliferation Cell Nuclear Antigen

LATS1: Large Tumour Suppressor Kinase 1

CYR61: Cysteine-rich angiogenic inducer 61

CTGF: Connective Tissue Growth Factor

mtROS: Mitochondrial reactive oxygen species

ChIP: Chromatin Immunoprecipitation

Chapter 1:

General Introduction, Gaps in Research and Objectives

1. Cancer and its epidemiology:

The simplest explanation defining 'cancer' is uncontrolled cellular growth. While this unchecked growth of cells at the surface might not seem a great deal, it stands as the second leading cause of mortality across the globe. As for the worldwide statistics, GLOBOCAN 2022 estimated 20 million new cases and 9.7 million cancer deaths in 2022[1, 2]. Globally, Asia accounted for 56.1% of cancer deaths and 49.25 of new cancer cases. While in India, the incidence of cancer cases is likely to increase from 1.46 million in 2022 to 1.57 million in 2025[3]. Breast cancer is currently the most common cancer diagnosed in women worldwide. An approximate 50% increase in the cancer burden has been predicted between 2020 and 2040. Since cancer is a complex system, the complexity can be attributed to the different characteristics that these tumour cells acquire over a period of time, such as sustained proliferative signalling and evasion of growth suppressors, among others highlighted [4] in **Fig. 1.1.**

These characteristics are acquired in various tumour types via distinct mechanisms and at different times during tumorigenesis. Another complexity of cancer systems lies in their heterogeneity, which often confounds drug resistance. Usually, when a heterogenous population is considered, Darwinian dynamics are applied where selection pressure can act on cells already primed with oncogenic mutations or cells with *de novo* variations[5, 6]. One of the major causes of intra-tumoral heterogeneity is genomic instability, which increases the probability of mutations in tumour cells. Another plausible source of genomic instability can also be epigenetics. Epigenetic modifications can alter the products expressed by affecting the specific transcription of cellular genes, hence augmenting intra-tumoral heterogeneity[7-10]

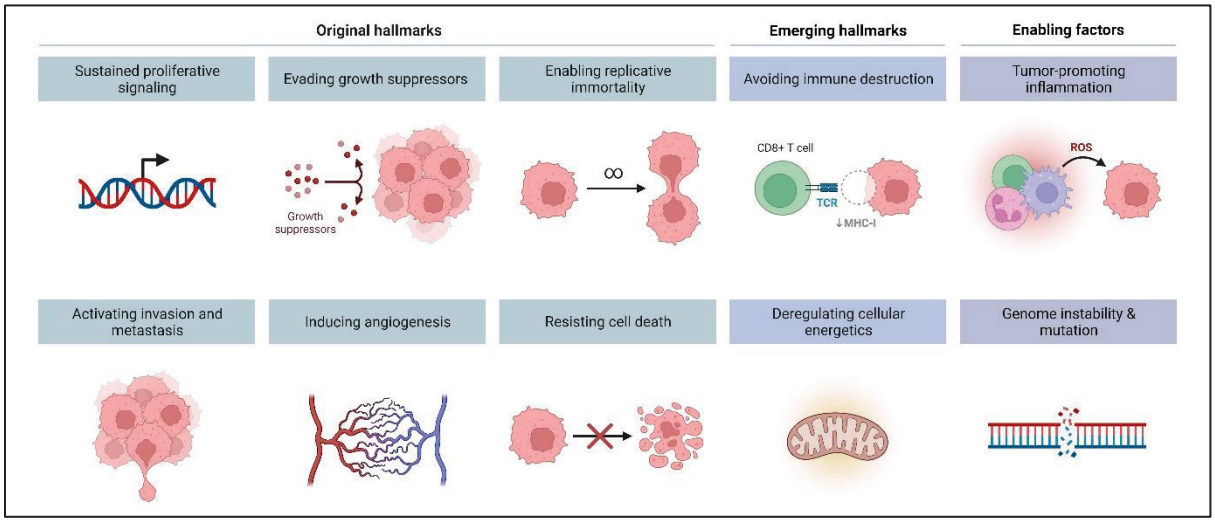


Figure 1.1 Illustration of hallmarks of cancer: Uncontrolled proliferation, metabolic reprogramming, immune evasion, inflammation, genomic instability, replicative mortality, resistance in cell death, invasion and metastasis, angiogenesis and evasion of growth suppressors are classical hallmarks of cancer progression.

Factors	Mode of action
Gene	Increases probability of tumour mutations
Epigenetics	Regulation of oncogenes via altered products
Microenvironment	Adaptation to different immune signals
Extra-chromosomal DNA (ecDNA)	Oncogenes on ecDNA are aberrantly but randomly expressed

Table 1.1 Factors influencing tumour heterogeneity and their mode of action

With epigenetics in play, and cells communicating with each other, only the Darwinian selection theory does not suffice how resistance sets in. Besides Darwinian selection, Lamarckian induction explains how transiently resistant (or tolerant) cells operate to become resistant. Therefore, in a tumour milieu, one can find both the forces acting together to contribute to drug resistance. Advances in cancer research have greatly augmented the treatment regimens; however, we are far from recapitulating the dynamic landscape that cancer

presents. A lot of efforts have been directed at understanding the molecular alterations of common cancers that have augmented the diagnosis and treatment however, the lack of the same for rare cancers has stilted the development of new therapeutic avenues. One of the rare cancers that needs extensive analysis is Osteosarcoma.

2. Our model- Osteosarcoma:

Although rare, Osteosarcoma (OS) affects approximately 3.4 million people annually. This tumour finds its origins in the mesenchymal tissues that form the bones. Among adolescents, it is one of the primary malignant bone tumours after brain tumours and lymphomas. Usually, OS sets in long tubular bones, such as the proximal tibia or the distal femur bones. OS is typically classified into central, surface tumours, and intramedullary with further subtypes under each group[11].

The most common type of OS that comprises of almost 80% of all cases affecting people within the first 20 years of their lives. This can be further divided into three types based on features of the cells that are involved: Chondroblastic, Fibroblastic, and Osteoblastic. Generally, histology labs require production of osteoid by tumour cells to confirm diagnosis of OS. Telangiectatic osteosarcoma, long for TOS, accounts for roughly less than 10 per cent of all cases of OS. The mean age of exhibiting features of TOS usually ranges from 15-20 years. Unlike OS, TOS is derived from mesenchymal stem cells or transformed osteoblasts and histologically appears as bone cysts. Interestingly, the majority of patients have a single lesion when they are first diagnosed with OS, and in most cases, metastasis manifests even before the actual onset. Approximately, 10-20% of patients have metastasis, with major sites as lungs (80-85%) followed by lymph nodes. However, these metastases do not show up since there are micrometastases, making diagnosis trickier than usual[12, 13].

2.1 Current treatment regimens:

Surgery- Two types of surgeries are carried out to resect OS: Amputation and limb salvage completely. Limb salvaging has been shown to increase the survival percentage by 80. Since OS presents as a single lesion often, surgery should completely remove the lesion else the recurrence rates can go as high as 25%. Recently, newer methods of removing tumour cells have been practised in patients with OS; removal of a tumour can lead to soft tissue defects and these defects can be corrected through reconstructive surgeries (prosthetic replacement, allogeneic bone transplantation, autologous bone transplantation, bone inactivation replantation) and/or ablation methods (temperature vs chemical). With the advent of ever-increasing technological advancements, computer-assisted tumor resection of OS is also being used now though it lacks some key hardware and software components to better assist[14].

Chemotherapy- In the late 1970s, chemotherapeutic interventions began for OS with high dose methotrexate reports by Jaffe. Simultaneously, Wang, Cortes, and Holland reported the use of Adriamycin. These were used as adjuvant therapy modules when metastases were not completely removed via surgery. At present, these drugs are used as neoadjuvants that have greatly impacted the systemic treatment of OS, with increased 5-year survival rate of OS. Presently, high dose methotrexate (HDMTX), Cisplatin (CDDP), ifosfamide (IFO), and Adriamycin (ADM) are used, in different doses and therapy combination sequences. Out of these, the MAP adjuvant chemotherapy is the first line of treatment given for OS[15]. It comprises of 6 administration cycles; Cycle 1-4 consists of Doxorubicin for day 1-2 as a continuous infusion for 48 hours or only a 4-hour infusion as a single dose, Cisplatin is administered for cycle 1-4 only on either day 1 and 2 for 2-4 hours or as a continuous 72-hour infusion for just day 1[15, 16]. Unlike doxorubicin, cisplatin, and ifosfamide which all function as DNA-damaging agents, methotrexate targets a key enzyme, dihydrofolate reductase (DHFR), that is involved in the folate cycle and is an essential component for the nucleotide

synthesis. In OS, methotrexate is given in high doses as it competitively inhibits the enzyme DHFR. To wear off the excess toxic effects of HDMTX, calcium folinate (leucovorin) is given. The side effects of MAP adjuvant therapy generally include the following: bone marrow suppression, neurotoxicity, gastrointestinal issues along with significant damage to the kidney and liver[17-19]. And even though Cisplatin is just given for the first 4 cycles, it has the potential to promote hearing loss in around 11% of total patients[20]. And single dose of ADM is known to cause permanent cardiomyopathy[21].

Apart from these existing chemotherapeutic therapies, vascular interventional therapy is also utilised in some cases to improve survival rates; this therapy administers chemotherapeutic drugs through arterial infusion with embolization of microspheres (formed either due to small systemic doses or large local doses)[22]. Currently, nanocarriers and various other nano delivery systems are under development for enhancing the therapeutic efficiency with minimal side effects.

Radiotherapy: Patients who do not respond well to neoadjuvant therapies, surgical resection or for whom the tumours are beyond the margins of selective resection are given local radiotherapy, especially for non-metastatic OS of the limbs. Reports by Ciernik et al showed that a high dose of radiation by proton therapy can augment the treatment[23]. However, OS is not potently sensitive to radiotherapies. Radiosensitizers are garnering much attention as they can increase the sensitivity of radiotherapy with minimal effects on normal tissues. In OS, ginseng polysaccharide and ionizing radiation have proved to be efficient[24, 25]. Additionally, as like with computer-assisted surgeries, advanced radiotherapy procedures such as proton radiotherapy, stereotactic radiotherapy are now being developed.

Immunotherapy: Another line of treatment that has proven efficacy in the last few years has been immunotherapy. Immunotherapy uses the patient's own immune system to fight cancer.

Immunotherapy in OS comprises adoptive immunotherapy, non-specific immunotherapy, specific immunotherapy, and immune-guided therapy[26]. In non-specific immunotherapy, cytokines present in abundance in the tumour microenvironment are used to modulate the immune system; Interleukin 2 has proven to be quite potent[27]. Apart from these, immune checkpoint inhibitors like CTLA-4, PD-L1 are also attractive avenues for immunotherapies[28].

2.2 Combination therapy: a promising tool

Despite these advancements, the majority of cancers are still treated with chemotherapeutic drugs. Especially in low and middle-income countries, targeted therapies such as immunotherapy are not viable options[29]; hence, platinum-based drugs such as Cisplatin and oxaliplatin, among others, are still prevalent. In India, clinical studies report the maximum usage of Cisplatin as the primary line of the drug due to its cost-effectiveness and availability[30, 31]. However, as mentioned earlier, increased toxicities such as nephrotoxicity, hepatotoxicity, and gastrointestinal toxicity are major problems of using these drugs. Hence, now, the majority of cancers are treated with a combination of drugs; as we can see, even for Osteosarcoma, we have a combination regimen to treat. As conventional monotherapies have higher toxicities associated with them, combination therapies specifically targeting cancer-inducing pathways work better. Although combination therapies can still have a comparably lower toxicity than monotherapeutic agents, they may produce cytotoxic effects on normal cells. Therefore, the sequence of drug administration and the dosages become important to any combination treatment.

Combination Drugs	**Cancer Type**	**Reference**
Paclitaxel	Ovarian Cancer Breast carcinoma Lung Carcinoma Melanoma Head & Neck	[32-34] [35] [36] [37] [38]
Paclitaxel & 5-FU	Gastric & Esophagogastric	[39]
UFT	NSCLC	[40]
Doxorubicin	Pleural Mesotheolima	[41]
Cyclophosphamide & Doxorubicin	Salivary Gland Carcinoma	[42]
Metformin	Lung adenocarcinoma	[43]
Bevacizumab	NSCLC	[44]
Tetra arsenic oxide	Cervical Cancer	[45]
Anvirzel	Breast, Colon, Prostrate, Melanoma and Pancreatic cancer	[46]

Table 1.2 List of some combination therapies with Cisplatin as a primary drug

Hence, the need for adjuvants that can exploit the differential vulnerabilities of cancer cells is at an all-time high. Small molecule inhibitors can be potent targets to circumvent the toxicity burden of conventional drugs. In this regard, Epidrugs is a growing avenue in the drug discovery field.

With respect to OS, its genetics are not enough to capitulate the diverse phenotypes observed in the OS landscape. Hence, we are now seeing a shift in research from classical genetics to a layered epigenetic mechanism. As explained earlier, aberrant epigenetic changes are involved in each stage of all cancer subtypes. Epigenetic changes in OS can be distinguished into three major components: Histone Modifications, Non-coding RNAs(ncRNAs), and DNA

methylation. While genetic mechanisms aggravating cancers are quite explored, recent advancements hint at the possibility of non-genetic alterations guiding resistance. This mainly involves mechanisms such as epigenetic reprogramming for acquiring resistance[47, 48]. Epigenetic alterations are reversible, and therefore, epigenetic therapies have the potential to modulate genes either by re-activating or silencing. In this ways, different cancer phenotypes can be reversed, effectively targeting cancer cells. These epigenetic alterations can also be induced due to external environmental stimuli, similar to genetic alterations. Hence, a comprehensive understanding of both the genetic and epigenetic programs is crucial to nailing the molecular progression of cancer.

3. Epigenetic regulation of Cancer: Basic mechanisms

Aberrant epigenetic changes in tumours have long been implicated in cancer development, progression, and resistance to chemotherapy[49]. In general, epigenetic events comprise of DNA methylation, modifications of Histone proteins, the readout of these modifications, chromatin remodelling, and effects of noncoding RNA. The expression or repression of the genome is controlled by the epigenome in conjunction with other regulatory factors, including transcription factors.

The tumor epigenome is characterized by global changes in DNA methylation and histone modification patterns as well as altered expression profiles of chromatin-modifying enzymes. Below **Fig. 1.2** illustrates the different key epigenetic modifications. Usually, aberrant DNA methylation at CpG islands leads to gene repression of tumor suppressor genes (like p53) that has been observed during the acquisition of drug resistance[50]. Such epigenetic changes result in global dysregulation of gene expression profiles leading to the development and progression of disease states.

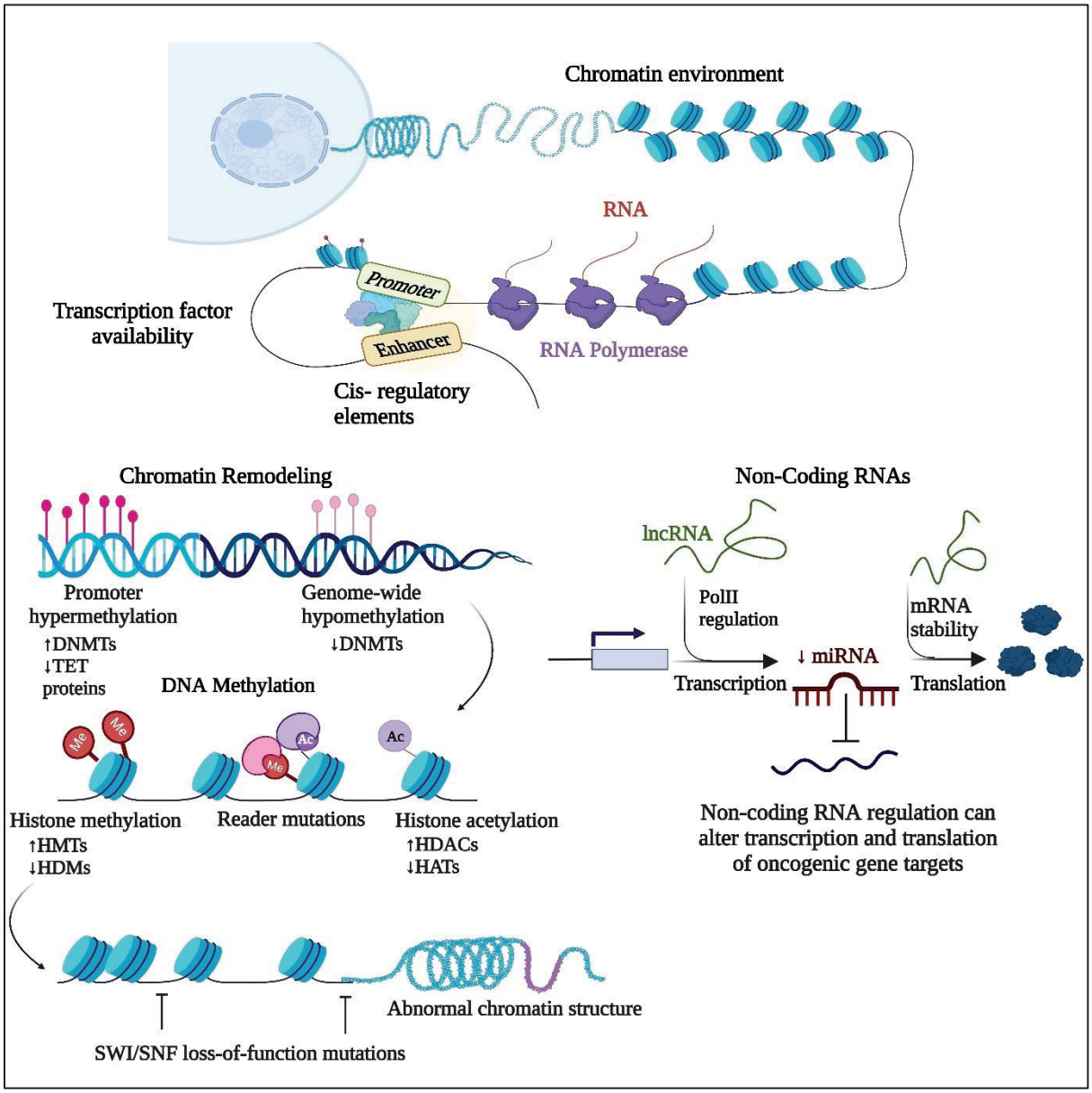


Fig. 1.2 Different Arms of Epigenetic Regulation (Mani N, Daiya A, Chowdhury R, Mukherjee S, Chowdhury S. Epigenetic adaptations in drug-tolerant tumor cells. Adv Cancer Res. 2023;158:293-335. doi: 10.1016/bs.acr.2022.12.006. PMID: 36990535)

3.1 Histone Modifications:

Another layer of epigenetic regulation is at the level of protein Histones. There exist four core histones- H2A, H2B, H3, and H4-that can be chemically modified. These modifications and the enzymes imparting these modifications can greatly contribute to different chromatin permissive states (open vs. closed), thereby influencing the expression of genes. Now, both

external and internal stimuli can cause these modifications to occur, and often, imbalance in this process can lead to aberrant cancer phenotypes that we observe. Apart from methylation, there exists other post-translational modifications such as biotinylation, sumoylation, and citrullination that can cause repression; crotonylation, butyrylation etc that causes activation.

When we talk about histone methylation, it usually takes place on the side chain of nitrogen atoms of either and/or lysine and arginine residues. Most methylation-specific modifications have been extensively defined for H3, but recent studies have shifted their focus to studying H4 as well. There exist multiple states for lysine and arginine methylation: mono, di and tri methylation, and different states contribute to gene expression differently. The commonly seen methylations are H3K4, H3K27, and H3K9. While H3K27me3 is a dynamically altering signature, H3K9me3 is more constitutively expressed[51]. Methylation of lysine is reversible with the presence of lysine demethylases such as LSD1.

Apart from methylation, acetylation of histones is also studied and is controlled by two classes of enzymes: Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs). Majorly three major families of HATs have been studied well that include: MYST (Tip60, MOF, MOZ, MORF, HBO1), p300/CBP, and GNAT (HAT1, GCN5, PCAF). HATs can also have non histones substrates too such as Rb, Myc, RUNX3, STAT3 [52] that they can directly regulate. Acetylation gives a negative charge to the positively charged lysine, thereby opening up the chromatin; they can also interact with bromodomain-containing complexes such as BAF complex to induce an open chromatin structure. Deacetylation of histones by the HDACs removes that negative charge thereby facilitating the closed chromatin structure and additionally preventing the accessibility of transcription factors. Four major families of HDACs are known: Class I (HDACs 1, 2, 3, and 8), Class II (HDACs 4, 5, 6, 7, 9 and 10) can shuttle between the nucleus and cytoplasm, Class III or sirtuins (SIRT1-7), which are NAD+ dependent and a different mechanism of action than their counterparts, Class IV with one

recently identified member, HDAC11[53]. As with HATs, HDACs can preferentially deacetylate non-histone substrates such as p53, HSp90, among others. Despite the recent advancements in understanding these different classes of Histone modifications in cancer, there remains a lot to be uncovered concerning disease stages and settings.

3.2 DNA methylation:

The mammalian DNA methylation machinery comprises of methyl CpG binding proteins (MBDs) and DNA methyltransferases (DNMTs), that cooperate to establish and maintain DNA methylation patterns. 5-methylcytosine (5mC) was one of the first identified epigenetic modulators in eukaryotes. This 5mC structure can prevent the accessibility of transcriptional factors to the binding sites of DNA ore recruit MBDs[54].

One of the cancer hallmarks is increased genomic instability. which is facilitated by DNA demethylation.. It has been observed that deletion or reduction of DNMT1 results in increased mutational burden, aneuploidy, and tumour progression. This does suggest that hypomethylation has significant effects on the fragility of chromosomes[55, 56]. And this loss may be coupled to the activation of oncogenes and/or transcription factors, further advancing the cancer ecosystem's complexity.

As most CpG islands are about 60-80% methylated, recent mapping techniques has helped us map to an extent the hypomethylation that sets in tumour cells; studies have divulged that DNA hypomethylation is usually present in blocks of 28 kb–10 Mb, covering about one-third of the whole genome. The promoters of Long interspersed nuclear element 1, or LINE1 for short, are also found to be hypomethylated at their CpG islands; this causes a permissive chromatin structure at alternative MET promoter, thereby activating oncogenes. It is well established that in several cancer types, such as melanoma, renal cancer[57]. In Osteosarcoma as well,

hypomethylation of gene Iroquois homeobox 1 (IRX1) causes its expression to be elevated and is linked to metastasis[58].

Where methylation in promoters renders chromatin closed, leading to gene repression, methylation in gene bodies is more positively correlated to gene expression[59, 60]. In Glioblastoma, studies have linked the elevated levels of MGMT with a high gene body methylation and unmethylated promoter region[61]. In cancers, hypomethylation in enhancers (intergenic and intragenic) can consolidate as binding motifs for transcription factors and change downstream gene expression. When we talk about DNA hypermethylation, IRAK3 is often found to be specifically hypermethylated in cancers such as colon adenocarcinoma[62]. Aberrant hypermethylation is also known to affect microRNAs[63], which can further affect the downstream signalling; in acute lymphoid leukaemia, hypermethylation silences miR-124a, activating the CD6-RB1 pathway, leading to poor survival rates[64]. Similarly, in gastric cancer, CpG hypermethylation of ncRNA nc866 also augments mortality[65]. This emphasizes that dysregulation in DNA methylation can either lead to the activation of oncogenes or the silencing of tumour suppressor genes, thereby contributing to the disease condition.

3.3 Non-coding RNAs:

Only 1-2% of the transcribed transcripts are translated to proteins. Considering this figure, we understand that the majority of the transcripts are non-coding. However, when compared to mRNAs, we see that these non-coding transcripts are present at low levels inside a cell. This does suggest two things: a) ncRNAs are involved in maintenance roles, and b) their abundance correlates with their conserved nature and hence is a good measure of their optimum function. ncRNAs can be further sub-classified as: microRNAs (miRNAs), long non-coding RNAs (lncRNAs), Piwi-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs)[66]. miRNAs regulate approximately 60% of genes and can interact with more than one mRNA.

miRNAs are dependent on DNA methylation but can also affect the DNA methyltransferases and other classes of histone enzymes, which shows their versatile nature. Studies in breast cancer have established the expression of individual miRNAs and their clinical features; in triple-negative breast cancer, elevated expression of miR-21, miR-210, and miR-221 is associated with poor survival[67-69]. Similarly, suppressor miRNA families in breast cancer, such as miR-200[70, 71] and miR-205[72, 73] family, mostly inhibit EMT induction, thereby reducing proliferation.

lncRNAs that are longer than 200 nucleotides with almost no potential for translation are often characterised as long non-coding RNAs (lncRNAs). lncRNAs greatly differ in expression based on cell types, development stage, and diseased condition. Some recent genomic studies have predicted around 15,000 lncRNAs in humans and are found to be more localized in the nucleus[74]. lncRNAs can also bind to transcription factors and can recruit chromatin remodelling complexes to promoters and alter the chromatin architecture; for example, lncRNA HOXA11-AS is known to bind to EZH2 to facilitate histone methylation that inhibits the transcription of the tumour suppressor gene p21[75]. Similarly, lncRNAs can act as a sponge for miRNAs to regulate the downstream gene expression; TUG1/miR-299/VEGF-A axis increases angiogenesis in glioblastoma[76] and AK131850/miR-93-5p/VEGF-A[76] is shown to promote differentiation, migration and tube formation of endothelial progenitor cells.

3.4 Targeting Epigenetic modulators for Cancer:

Since the etiology of cancer is quite convoluted, epigenetic dysregulations add a complex layer to them. As discussed above, targeting epigenetic modifications, there is a possibility of the cancer phenotype to revert to the parental phenotype. However, the challenge again lies in capitulating these modifications across different cancer subtypes alongwith stages. Until now, efficiency of these epigenetic drug or ‘epidrugs’ has been seen only in hematological

malignancies[77, 78]. Currently, use of DNA methylation inhibitors and histone deacetylation inhibitors is utilised. **Table 1.3** shows some of the epigenetic drugs in trials for various cancers.

Cancer	Epidrug	Phase	Current Status	NCT
CML	Azacitidine	III	Completed	NCT01350947
Prostrate	Azacitidine	II	Completed	NCT00384839
AML	Azacitidine+venetoclax	II	Recruiting	NCT03466294
Pancreatic	Azacitidine	II	Recruiting	NCT01845805
Glioma	VPA+Levetiracetam	IV	Recruiting	NCT03048084
High Grade Glioma	VPA+TMZ+Radiation	II	Completed	NCT00302159
Advanced pancreatic adenocarcinoma	PRI-724+Gemcitabine	I	Completed	NCT01764477

Table 1.3 Epigenetic drugs in trials for various cancers

Apart from being used as single agents by FDA, studies are trying to work with their combination, especially for solid cancers, to increase their efficacy. For instance, in non–small cell lung cancer (NSCLC), 65 patients were given combined low doses of 5AC (DNMTi) and the entinostat (HDACi), which gave a better response lasting almost 4 years[79]. Another rapidly emerging field is to combine these epidrugs with conventional drugs or other therapies; for instance, HDACi SAHA when given with paclitaxel has shown better overall progression free survival in patients with metastatic NSCLC[80]. Some groups are also working towards combining epigenetic therapies with immunotherapy. In the above mentioned trial carried out in 65 patients, five patients who were given epigenetic therapy were also subsequently given anti-PD-1 and PD-L1[81]. As compared to 20% of

patients who only had PD-L1, the five patients who had epigenetic therapy prior had progression-free survival greater than 24 weeks unlike the ones who received just the immunotherapy[81, 82]

Small new molecules are also being tested for these epigenetic mediated therapies. EZH2 is one regulators that is now being studied extensively, for which inhibitors are introduced in clinical trials, mostly for hematopoietic tumors. For mixed-lineage leukemia translocations in AML, DOT1 (disruptor of telomeric silencing–1) inhibitors are there in clinical trials for AML[83-85]. Interestingly, small molecule inhibitors for BRD4 are now being taken for clinical trials[86, 87].

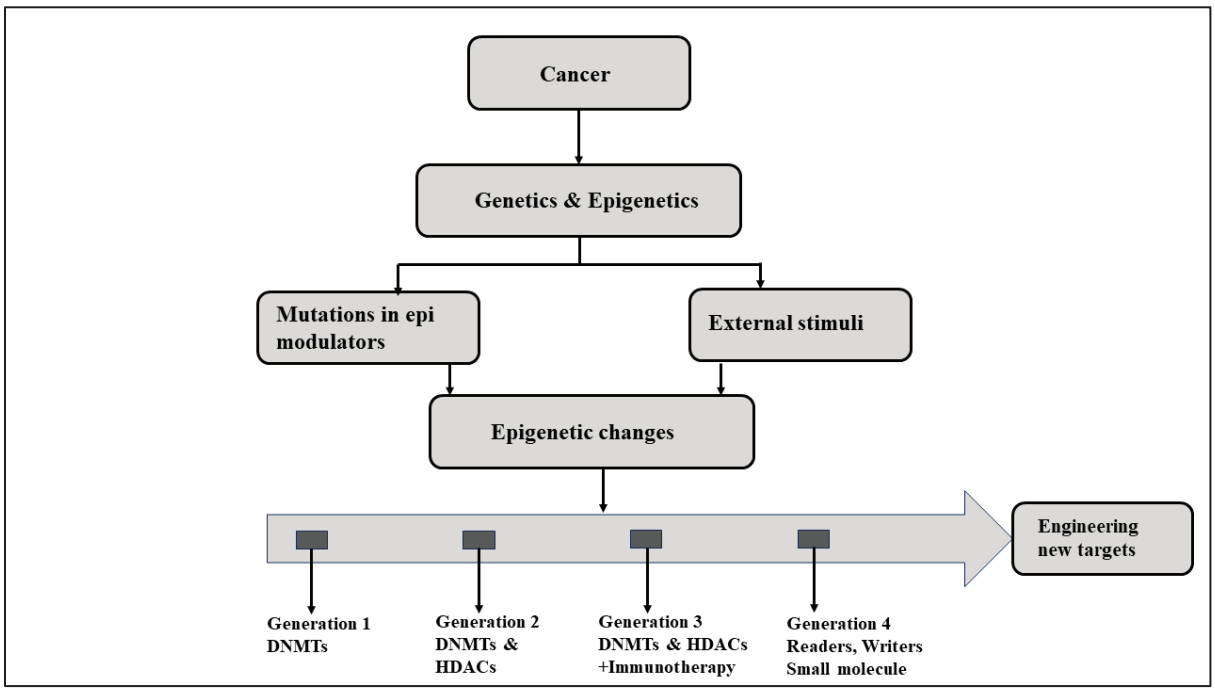


Fig. 1.3 Epigenetic targeted therapies based on current and future prospects

All these datasets substantiate the shift seen from single-agent chemotherapies to combining drugs to circumvent this growing problem of drug resistance and reversing epigenetic abnormalities.

4. Epigenetic Signatures of OS:

Histone modifications are carried out by histone-modifying enzymes that allow covalent modifications to the NH2 terminus of histone tails, called post-translational modifications. This change either condenses or decondenses the chromatin, leading to changes in gene expression. While Histone methylation has been extensively studied, the dynamicity of these modifications renders them difficult to pinpoint at each step. Some studies have shown Lysine-specific demethylase (LSD1) to be overexpressed in OS tumours, and using inhibitors of LSD1 can lead to a reduction in cellular proliferation[88]. Similarly, the p53-guardian of the genome is also involved in OS formation. Studies on cell lines have found the stabiliser of p53, $P14^{ARF}$, [89]to be methylated in its promoter site; further treatment with histone-demethylating agents reversed the activity of $P14^{ARF}$. Similarly, increased expression of histone methyl transferases such as enhancer of zeste homolog 2 (EZH2)[90], G9a[91], NSD3[92] is also seen in Osteosarcoma. Another Histone methyltransferase, SUV39H2, when expressed in high levels makes cells resistant to chemotherapy and is also known to dribble with LSD1. H3K27me3 levels are often dynamic as mentioned above and this contributes significantly to cancer progression. While few reports have found increased DNMT expression leading to hypermethylation of promoters, methylation of cell cycle genes via lncRNAs such as HOTAIR also make up for the epigenetic signatures in OS. Study by Parker et al. defined the promoter hypermethylation in 489 pediatric tunour samples; they found 32 genes to be differentially methylated between pediatric tumours and SPRY2 gene was a top hit[93]. In one of the studies, KDM6B in OS biopsies was found to be upregulated, and levels of H3K27me3 were high in control OS cells. One of the genes they found to be downregulated was Lactate dehydrogenase A (LDHA) in control OS cells[94].

Recently, Piao et al. showed the relation between decrease of histone 4 lysine 20 trimethylation (H4K20me3) and poor prognosis in OS; in comparison to normal samples, low levels of

H4K20me3 were seen in both OS tissue samples and OS cell lines Consequently, mRNA expression of H4K20me3's catalyst, SUV420H2, was also downregulated in OS cell lines[95].

Apart from readers, writers and erasers, lncRNAs have also been well-implicated in OS biology. For instance, high levels of PVT1 were correlated with poor prognosis and enhanced EMT in OS[96]. Additionally, PVT1 also acts as a sponge for miR-486. Zhu et al. showed the lncRNA MEG3 augments OS doxorubicin (DXR) resistance by enhancing the expression of AKT2, that can be inhibited overexpressing miR-200b-3p[97].

All of these epigenetic regulatory mechanisms regulate different, occasionally overlapping signalling pathways that cause tumor development or progression in Osteosarcoma. Hence, understanding these epigenetic modulations alongside their effects on signalling pathways and vice versa is very crucial.

5. Signal Transduction and Epigenetics:

Cells respond to external stimuli by activating unified signals that traverse through cytosol before reaching the nucleus and imparting its effect. The precise activity of these molecular signals and their integration is a marvellous aspect of biology. These pathways are responsible for turning these signals into specific transcriptional states, and those states often define the corresponding gene activation or repression at a particular locus. Although different signal transduction pathways and mutational changes in the components of those pathways have already been well-studied in cancers, much less is known about the crosstalk of these signalling pathways with changes in chromatin structure or vice-versa. Some more common yet diverse signalling pathways, such as JAK-STAT, MAPK, WNT, [26, 98, 99]have already been studied for their interaction with epigenetics. For instance, MAPKs are popular kinases that will work through phosphorylation. In regard to meddling with epigenetics, it is now known that MSK1/2 can cause the phosphorylation of H3, and HMG-14 chromatin associated protein[100]. This

causes phosphorylation at Ser28 of H3, which in turn does not allow PcG group of proteins to deposit H3K27me3 repressive histone marks, thereby activating the targeted genes. Furthermore, this change also causes acetylation to be increased at K27 thereby further augmenting the transcriptionally active state. Similarly, HDACs are involved in differentiation; interestingly, HDAC1/2 are involved with WNT signalling in the process of oligodendrocyte formation. In precursor cells, transcription factor TCF7L2 acts as a repressor bound to Beta catenin. As differentiation progresses, HDAC1/2 actually compete with Beta catenin for binding to TCF7L2, to activate oligodendrocyte specific genes[101].

Another highly conserved pathway is JAK-STAT, which is involved in regulating apoptosis, proliferation. Recent reports in Drosophila prove that PRC1 represses expression of Upd ligands while nucleosome remodelling factor (NURF) in Drosophila testis has been seen to regulate JAK-STAT to maintain germ stem cells and cyst progenitor cells[102].

Looking at the inflammatory environment of any cancer, it is interesting to note that epigenetics also can have a role in activation of such inflammatory responses. Natoli et al. interestingly found that the expression of Jmjd3 histone demethylase (specific for H3K27me3) was upregulated in macrophages when exposed to inflammatory cytokines. Subsequently, they found three κB binding sites in the promoter of Jmjd3, suggesting that the inflammation-activating NF-κB pathway affects Histone methylation directly. Thus, activation of a specific histone demethylase is how NF-κB exerts its functions[103, 104].

If we go the other way, miRNAs are also known to be key regulators of several signalling pathways. Additionally, these miRNAs are also known to interact and modulate different epigenetic factors. This suggests that miRNAs could be a building bridge between cellular signalling and epigenetic modulation. For instance, miR-145 is known to be involved in stem cell differentiation[105]; while miR-145 can cause OCT4 to get silenced post-transcriptionally,

it itself is negatively regulated by OCT4[106]. It is also known that miR-145 can epigenetically be silenced via DNA hypermethylation[107]. All these findings suggest a complex regulatory system exists for cells to function in more than one way.

Emerging evidence, as otherwise listed here, proves that both signal transduction and epigenetic regulation are not separate concepts. And that the relationship of the two is forming a new framework of gene regulation. Studying both mechanisms can help us better understand the cancer niche to find better therapeutic targets.

5.1 Epigenetic regulation of Hippo signalling:

Hippo signalling has been extensively studied for the last 2 decades now. In mammalians, Hippo pathway is a conserved pathway involved in maintain the organ size and stem cell niche[108]. There are several key components of Hippo pathway as shown in **Fig.1.5** YAP/TAZ are transcriptional co-activators that bind to TEAD1-4 to regulate the expression of genes. Mechanical cues, hypoxia, cellular density, cell polarity, GPCRs, are some of the other key upstream signalling components. Several cancers have correlated upregulated YAP levels to poor prognosis[109-113]. YAP/TAZ play a role in maintaining the cancer stem cell niche in the tumour milieu, explaining its heterogeneity. YAP/TAZ favours communication between the tumour cells and the stromal cells of the microenvironment via the secretion of AREG, EGF-like growth factor.

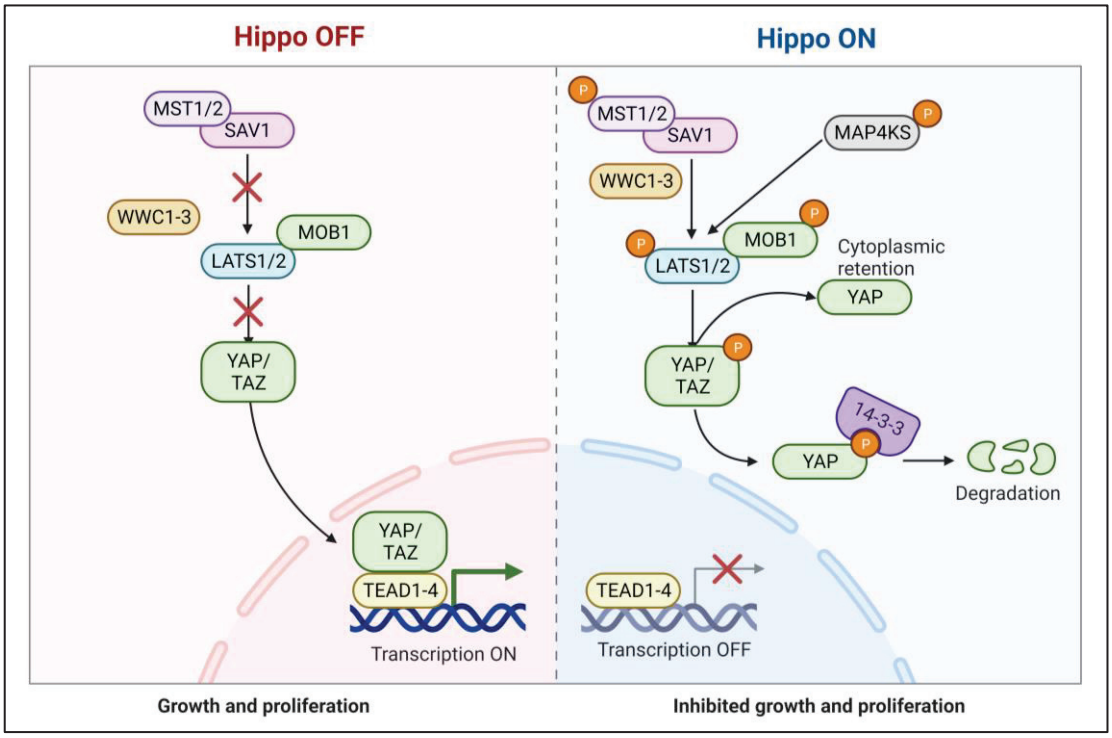


Fig. 1.4 Core Hippo Pathway in Mammals. Evolutionarily conserved pathway involved in regulating cell proliferation and stem cell renewal. Kinase cascade where MST1/2 and SAV1 forms complex to activate LATS1/. LATS1/2 in turn, phosphorylates YAP/TAZ. When not phosphorylated, YAP/TAZ translocate into the nucleus and induce the expression of genes involved in cellular proliferation.

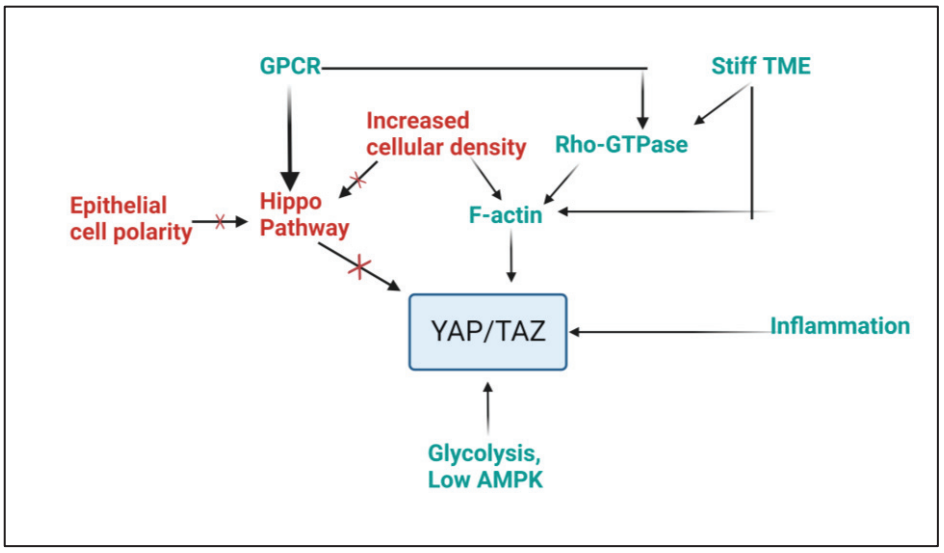


Fig. 1.5 Illustration of different inputs regulating YAP/TAZ activity in tumour

Additionally, it is now being seen that YAP/TAZ might actually interact with distal enhancer/silencer elements to propagate malignant properties. For instance, AP-1 factors are known to be activated by enhancer elements bound by YAP/TAZ[116]. It has already been established that YAP/TAZ activation protects the cell against DNA-damaging agents, it also promotes resistance to therapies targeting RAF and MEK inhibitors[117] in tumour cells that harbour mutations such as BRAF and KRAS, among others. Interestingly, recent studies have highlighted how there is an increase in the polymerization of actin stress fibres during the acquisition of resistance along with increased nuclear expression of YAP[118]. This substantiates that changes in ECM can make cells resist molecular treatments. While some studies suggest that YAP cooperates with with TEAD to promote proliferation in KRAS-deficient PDAC cells, some studies mention the deficiency of YAP can sensitize KRAS-deficient PDAC cells to pan-RAF treatments[119]. This highlights that YAP/TAZ induces resistance in more than one way and has bypass mechanisms that may or may not be ubiquitous across cancer subtypes.

Not many reports at present give a comprehensive understanding of the involvement of epigenetic modulators in regulating Hippo pathway, but recent reports have highlighted the importance of KDM4D (histone demethylase) on cell cycle activity of cardiomyocytes. Another study reveals the inactivation of Hippo pathway via DNA methylation; increased DNA methylation at the promoters of NF2 in Hepatocellular carcinoma tissues, resulting in decreased expression[120]. Apart from these, miRNAs have been extensively implicated in regulating the different components of Hippo signalling, as shown in **Table 5.1 and 5.2.** miRNAs can function both as tumour suppressors and oncogenes and are now being looked at as potential biomarkers since some of these are found to be freely circulating in the cells.

miRNA	Target	Tumour Type	Ref
miR-338-3p	YAP, TAZ	Liver Cancer	[22]
miR-181c	core kinase cassette, i.e. MST1, LATS2, SAV1 and MOB1/YAP/TAZ	Pancreatic cancer	[121]
miR-137	CUL4A	Gastric cancer	[122]
miR-9	CUL4A	Gastric cancer	[122]
miR-363	LATS2	Ovarian cancer	[123]
miR-21	YOD1	Liver cancer	[124]
miR-31	LATS2	Esophageal cancer	[125]
miR-149−5p	SAV1 and MST	Ovarian cancer	[126]
miR-135b	LZTS1, FBXW1A, NDR2 and LATS2	Lung cancer	[127]
miR-93−5p	CDHF14 and LATS2	Gastric cancer	[128]
miR-129−5p	YAP and TAZ	Ovarian cancer	[129]
miR-130a	YAP	Liver cancer	[130]

Table 1.4 Oncogenic miRNAs regulating Hippo pathway components in different cancers

miRNA	Target	Tumour Type	Ref
miR-200a-3p	YAP1	Breast cancer	[131]
miR-125a	LIFR	Breast cancer	[132]
miR-424	CDK1 and LATS1	Breast cancer	[133]
miR-874−3p	YAP and TAZ	Colorectal cancer	[134]
miR-375	YAP, TEAD4 and CTGF	Gastric cancer, Liver cancer	[135, 136]
miR-15a	YAP	Gastric cancer	[137]
miR-16−1	YAP	Gastric cancer	[137]
miR-138−2-3p	YAP1	Laryngeal cancer	[138]
miR-4269	YAP1, TEAD1 or TEAD4	Gastric cancer	[139]
miR-1343−3p	YAP1, TEAD1 or TEAD4	Gastric cancer	[140]
miR-186	YAP1	Liver cancer	[141]
miR-9−3p	TAZ	Liver cancer	[142]

Table 1.5 Tumour suppressing miRNAs regulating Hippo pathway components in different cancers

Interestingly, first defined in Drosophila, YAP/TAZ is found to interact with SWI/SNF complex, which is an ATP dependent chromatin remodelling complex[143]. In mammalian systems, TAZ is important for controlling mammary epithelial cell lineage switching between luminal and basal cellular fates; TAZ deficiency in basal cells causes it to switch to luminal

cell fate whereas nuclear expression of TAZ in luminal cells makes them more basal type. In this regards, co-immunoprecipitation assays have revealed the interactions between TAZ and SWI/SNF complexes[144]. Furthermore, in HSCC patients, SWI/SNF mediated activation of YAP is correlated with poor prognosis[145]. Another instance of how YAP might facilitate chromatin changes is through its interactions with the NuRD complex. The NuRD complex comprises of both ATP-dependent chromatin remodelling with histone deacetylase activity. In this way, NuRD causes the compaction of nucleosomes around regulatory regions to restrict accessibility to transcription factors. Human YAP/TAZ/TEAD have been shown to interact with the NuRD complex to repress target gene activity[146]. Although the NuRD complex can activate target transcription major instances of YAP/TAZ/TEAD are all repressive.

However, how the Hippo pathway specifically gets regulated via epigenetic modulators other than miRNAs is still elusive, but ongoing investigations are sure to give us a more comprehensive understanding of how these epigenetics and Hippo signalling can work in conunction.

6. Gaps in Research & Objectives:

Cancer is a complex system, and heterogeneity in cancer is one major force that often confounds treatment. In a clinical setting, one particular drug or one specific dose may not be effective for all the cancer subtypes. Similarly, different cancers may depend on specific epigenetic alterations for survival, while others may be sensitive to the same. Hence, identifying the most important epigenetic alterations facilitating the survival OS cells especially needs a comprehensive analysis. And since many different epigenetic alterations exist at the same time, we need to examine the most critical one which can facilitate the sensitization of our chosen model of cells: Osteosarcoma. While epigenetics may be the driver, the molecular landscape is often governed by the changes in tumour suppressors and/or

oncogenes. Hence, understanding and pinpointing a molecular partner that can be epigenetically targeted or can itself be part of epigenetic machinery is crucial to identify. While not always one singular modification or pathway may be involved in promoting survival of tumour cells, it is imperative to pinpoint the major pathway that could be targeted before the small subpopulation of cells adapts too well. Additionally, how the parental drug affects the epigenome, especially the histone modifications is quite elusive. Furthermore, functional crosstalk between the epigenetic changes post-drug treatment with the pertinent signalling pathways also needs a thorough investigation to design appropriate combinatorial therapies that can advance the benefits of current therapies in Osteosarcoma.

Therefore, based on our extensive literature review and the above-mentioned loopholes, we have designed the following objectives:

1. Exploring the epigenetic adaptations and its functional implications in tumour cells exposed to conventional chemotherapeutic agent
2. Analysing the crosstalk between selected epigenetic alteration(s) and key signalling molecule(s) post drug exposure.
3. Exploring an appropriate combination treatment to effectively sensitize tumour cells to conventional chemotherapeutic agent

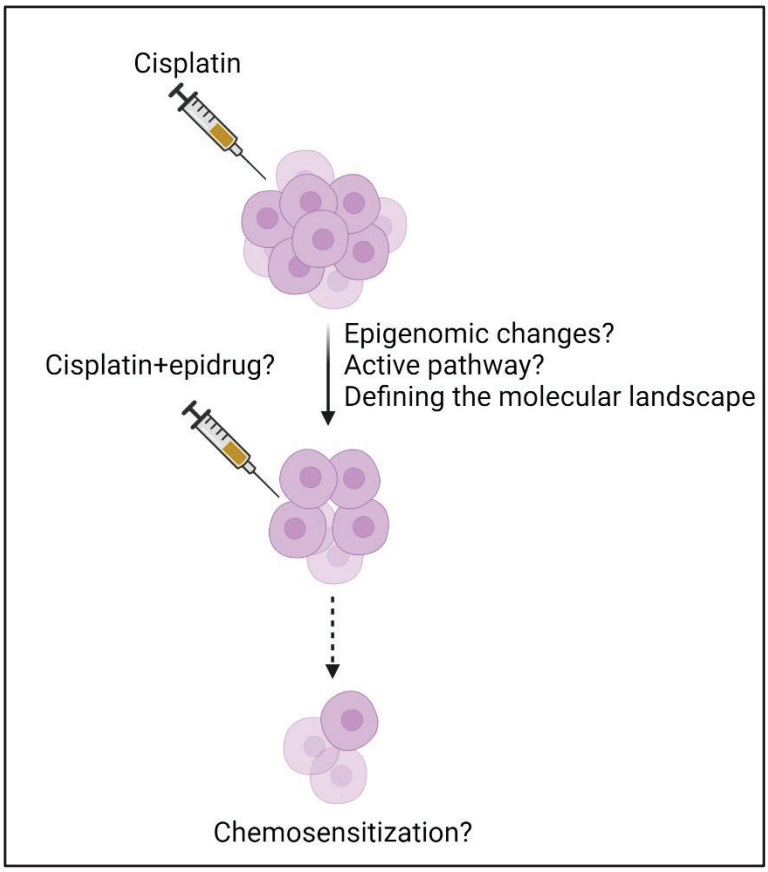


Fig.1.6 Schematic of Gaps in Research

Chapter 2:
Materials & Methods

2.1 Chemicals and reagents:

Cisplatin (CDDP; 232120-50M) and EZH2i GSK-126 #5005800001 were obtained from Merck; 2′,7′-dichlorofluorescin diacetate (DCFDA, #D6883), TRI reagent® (#T9424), RIPA (#R0278), Bradford reagent (#B6916), Propidium iodide (PI; #P4864), DAPI (#D9542), Protease Inhibitor cocktail (#P8340-1ml), and Verteporfin (#SML0534-5MG) were purchased from Sigma-Aldrich; N-Hydroxy-N'-phenyloctanediamide (SAHA) #H1388 was purchased from TCI; N-acetyl cysteine (NAC,#47866) and MTT (#33611) were procured from SRL; Annexin V, FITC-Conjugate (#A13199), and secondary antibodies Alexa fluor Plus 555 #A32732 & Alexa fluor Plus 488 #A32733 were procured from Thermo Fisher Scientific; JC-1Dye (#T3168), MitoSox (#M36008), Lipofectamine 3000 (#L3000-001), and MAGnify™ Chromatin Immunoprecipitation System (#492024) were from Invitrogen; Clarity™ Western ECL Substrate (#1705061), iTaq Universal SYBR Green Supermix (#1725121), and cDNA synthesis kit (#1708891) were obtained from BioRad; Secondary antibodies: anti-mouse (#7076S) and anti-rabbit (#7074P2) were from CST; siRNA YAP (siRNA ID #107951) and scrambled siRNA (siRNA ID #32-6976). Luciferase plasmid (8XGTIIC) was a gift from Stefano Piccolo (Addgene, #34615). Minimum Essential Medium Eagle (MEM) (HiMedia, #AL047S); Luciferase Assay Kit (#E1500) was purchased via Promega. Phalloidin-iFluor 488 (#Ab176753) was procured from Abcam.

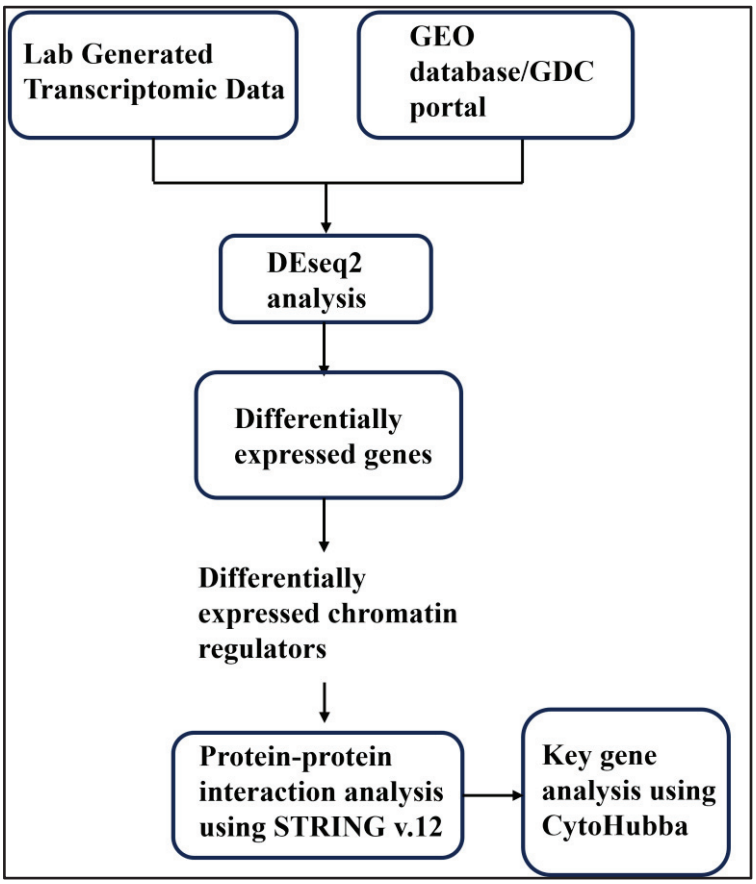
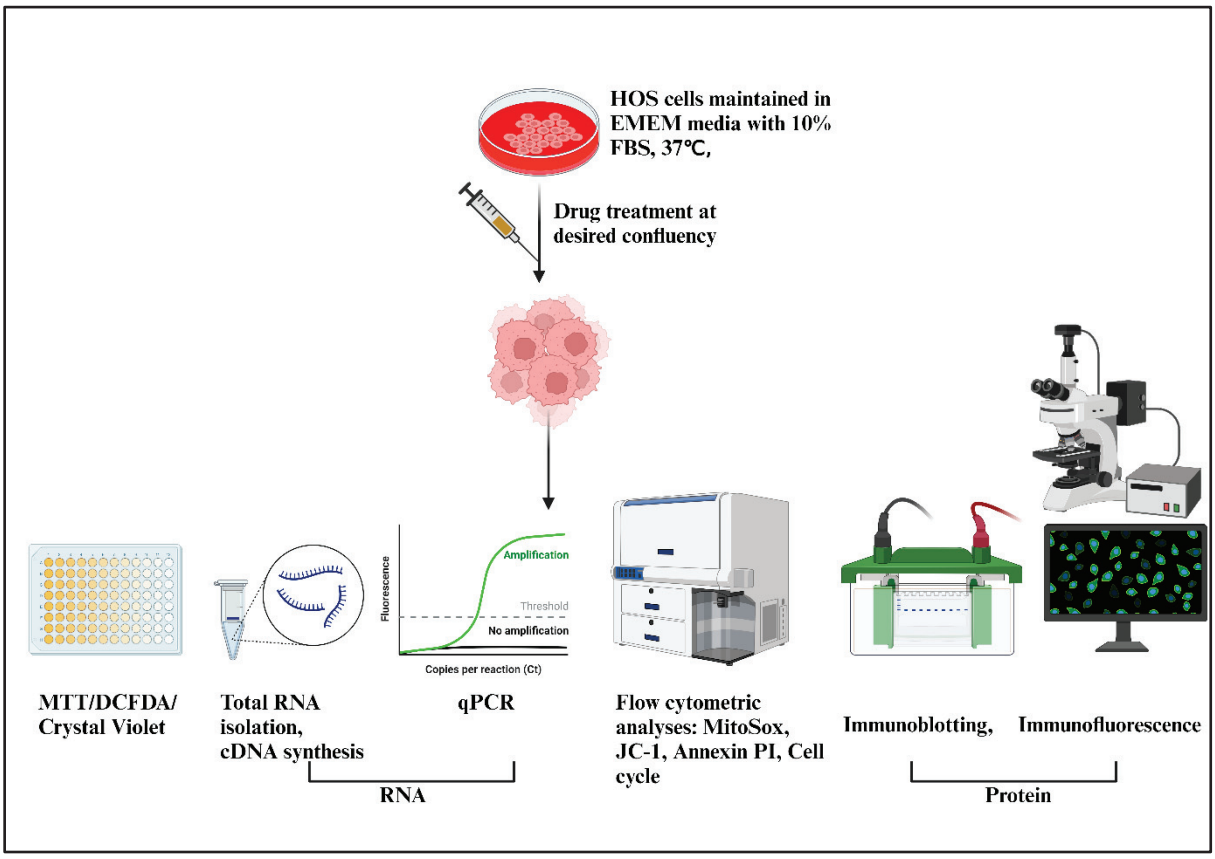


Fig. 2.1 Flowchart depicting the methodology adopted for Key Gene analysis



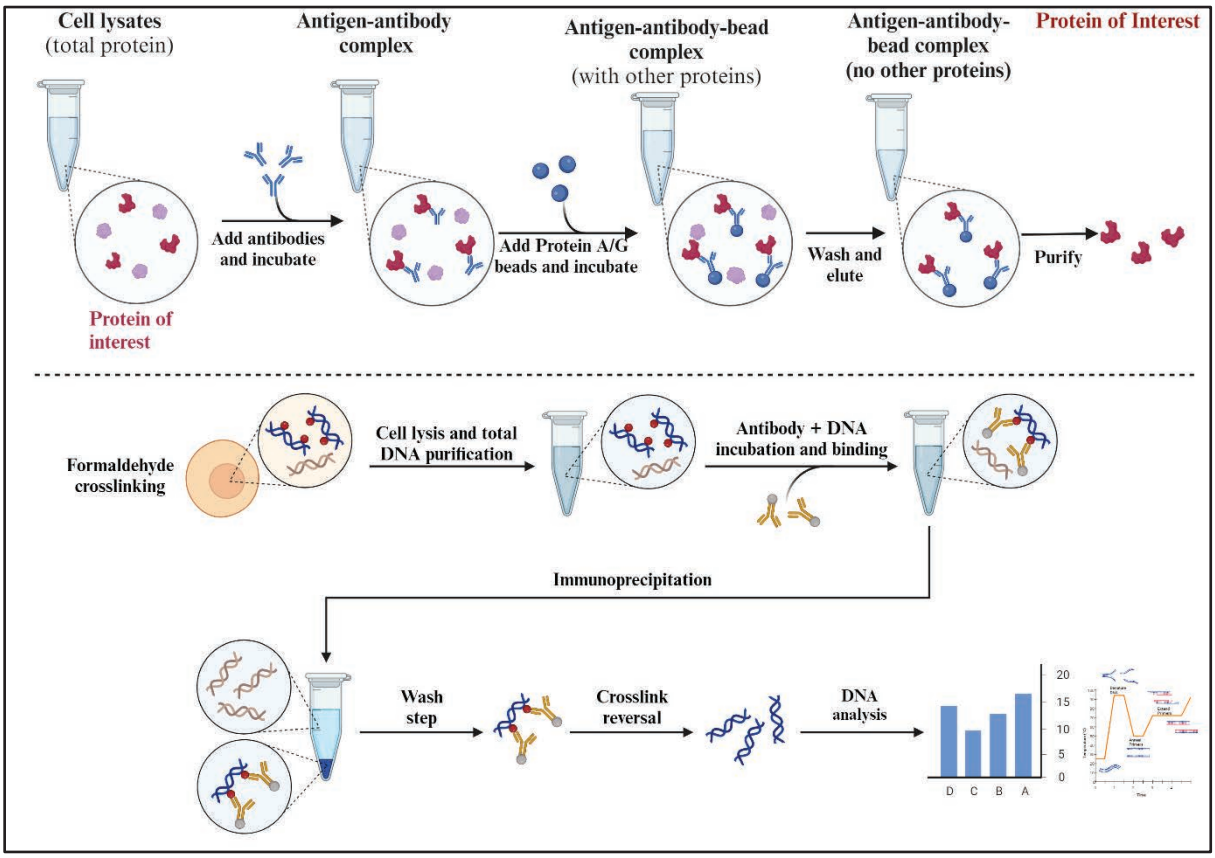


Fig.2.2 Flowchart depicting the various experiments performed *in-vitro*

2.2 Methods:

2.2.1 Cell culture:

Human Osteosarcoma (HOS-CRL-1543) cell line used in this study was procured from NCCS, Pune, India. Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution at 37°C, 5% CO2 levels. Typically, the cells were grown to 60–70% confluency, rinsed in phosphate-buffered saline (PBS) and seeded into a fresh medium before being exposed to designated drug treatment(s).

2.2.2 Viability Assay:

To evaluate the viability of cells, an MTT assay was performed at different concentrations of CDDP in the culture medium. Approximately 6000 cells per well were seeded in a 96-well plate. As cells gained their morphology, they were treated with different concentrations of CDDP for different time points (24h, 48h, 72h). MTT (at a final concentration of 1mg/ml) was added to the plate and incubated for 3h. To solubilise formazan crystals, DMSO was used. Differential filters of 630nm, along with a reading at 570nm for formazan crystals, were taken using a Multiskan GO microplate spectrophotometer. The following formula was used to calculate percentage cell viability = (mean absorbance value of Cisplatin treated cells)/ (mean absorbance value of the control) x 100.

2.2.3 Analysis of total reactive oxygen species:

Total intracellular ROS levels were measured using 2, 7-dichlorofluorescein diacetate (H2DCFDA). This widely used assay measures the generation of H_2O_2. H2DCF-DA enters the cell passively, reacting with generated ROS to form one highly fluorescent compound, dichloro-fluorescein (DCF). For this, around 6×10^3 cells per well were seeded and were treated with CDDP for 24h. Two hours before the drug treatment, ROS quencher (NAC) was added to inhibit basal ROS. Cells were washed with 1X PBS and incubated in 20µM DCFDA for 30

minutes at 37°C. Fluorescence readings were taken at excitation: 485nm, and emission:530nm using a microplate reader (Fluoroskan Ascent).

2.2.4 Crystal Violet Assay:

Another qualitative method to assess cell viability is through crystal violet assay. Dead cells lose their adherence, and crystal violet dye which binds to DNA and proteins, can be used to distinguish dead and viable cells. Cells undergoing apoptosis do not take up this dye, thereby reducing the amount of crystal violet in a culture. Cultured cells are given a 1X PBS wash before incubating them with 0.5% crystal violet solution per well for 10 minutes. Post this, cells are thoroughly rinsed with MilliQ water to remove the nonspecific staining. The plates are allowed to dry for some time before visualising them under a microscope.

2.2.5 Total RNA isolation, cDNA synthesis and PCR studies:

Total RNA extraction was performed using TRIzol reagent. Post treatment, 1.2% Agarose gel was run to visualise the integrity of the RNA while simultaneously RNA was quantified. After this, DNase I treatment was given to the samples, and this RNA first strand cDNA. cDNA synthesis was done according to the manufacturer's protocol: iScript cDNA synthesis kit. Templates were amplified using gene-specific primers for YAP, CYR61, CTGF, EZH2, LATS1, PCNA, CCNA2, promoter primers: YAP, LATS1, CCNA2. β-Actin was designated as a housekeeping control. Amplicons were detected using SYBR Green Supermix through Quant Studio 3.0 (q-PCR system by Applied Biosystems). The respective sequences of forward and reverse primers have been enlisted below in the form of a table (Table 3.2). The relative mRNA expression was calculated using Pfaffl's method.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Tm (°C)
YAP	TAGCCCTGCGTAGCCAGTTA	TCATGCTTAGTCCACTGTC TGT	55
CTGF	CAAGGGCCTCTTCTGTGACT	ACGTGCACTGGTACTTGC AG	57.2
CYR61	ATGGTCCCAGTGCTCAAAGA	GGGCCGGTATTTCTTCAC AC	55.8
EZH2	GCTTCCTACATCGTCGTAAG TGCAA	GCTCCCTCCAAATGCTGG TA	56
LATS1	GTTAAGGGGAGAGCCAGGT CCTT	TCAAGGAAGTCCCCAGGA CTGT	52.6
PCNA	TCACAGGGCAGTGTCTTCAT T	TCACAGGGCAGTGTCTTC ATT	56.2
CCNA2	GACTTAGCTGCTCCAAACAG	GCTTTGTCCCGTGACTGT GT	60
YAP-P	GTTGCGGCTTCCAGTGACTA	AAGCCGCGAGGATAGATT GG	66.3
LATS1-P	CAACGATCCCATCCCACACT	TCTGTCAACCGCATCCGT AG	66.6
CCNA2-P	ACTGAAAAACGTGCCCCAG A	TTTGGGTTGCCCAGCCTT TA	60
HDAC5	CCCGTCCGTCTGTCTGTTAT	CTGACATCCCATCTGCCG AC	63.8

HDAC9	**CTGGAGCCACTTGCAGAAGA**	**AGGCTGGCTCCTCTTCCATA**	**64**
GAPDH	**GCACCGTCAAGGCTGAGAAC**	**TGGTGAAGACGCCAGTGGA**	**50**
β-Actin	**CCACCATGTACCCTGGCATT**	**CGGACTCGTCATACTCCTGC**	**62.1**

Table 2.1 List of primers used for PCR

2.2.6 Immunoblotting:

As described elsewhere, the immunoblotting procedure was followed. Modified RIPA buffer was used to lyse the treated cells. After that, total protein was measured using Bradford reagent. Loading dye (5X) was added to prepare the lysates, and lysates were heated at 100°C for 10min. Lysates were then run in SDS-PAGE gel and transferred to the PVDF membrane. Membrane blocking was done with 5% skimmed milk or 3% BSA or 5% skimmed milk. Membranes were then probed/re-probed with specific primary antibodies. The secondary antibodies were horseradish peroxide-conjugated; hence, enhanced chemiluminescence detection (ECL) system was used per the manufacturer's protocol. As and whenever required, the blots were cut accordingly to probe with various antibodies against proteins of different molecular weights. The antibodies used in the study are mentioned in the table below. ImageJ software was utilized for quantification.

Antibody	**Catalogue Number, Manufacturer's Name**
YAP/TAZ	#8418, CST;
YAP	63.7 #sc-101199, SCBT
p21	#2947S, CST
CYR61	#14479, CST
MOB1	#13730, CST
PhMOB1	#8699, CST
H3K27me3	#9733S, CST
H3K9me3	#13969S, CST
H3K4me3	#9751S, CST
H3K27ac	#8173, CST
H3K9ac	#9649, CST
EZH2	#5246S, CST
LATS1	#3477T, CST
CTGF	#88641, CST
PCNA	#13110S, CST
GAPDH	G-9 #sc-365062, SCBT
β-Actin	#BB-AB0024, BIOBHARATI

Table 2.2 List of antibodies used in the study

Antibody	**Catalogue Number, Manufacturer's Name**
Secondary Anti-Mouse	#7076S, CST
Secondary Anti-Rabbit	#7074P2, CST
Anti-Mouse IgG Secondary Alexa Fluor 488	#A32723, Invitrogen
Anti-Rabbit IgG Secondary Alexa Fluor 555	#A32732, Invitrogen
Anti-Rabbit IgG	#I5006, Sigma

Table 2.3 List of secondary antibodies used in the study

2.2.7 Annexin-V/PI apoptosis assay through flow cytometry:

Annexin-V/PI is one way to identify the percentage of necrotic and apoptotic cells in a given population. Annexin-V binds to phosphatidylserines found in the inner side of the cellular membrane; this gets flipped up during the early stages of apoptosis. Propidium Iodide usually enters the cell when the membrane is ruptured and binds to the DNA. For this, cells were seeded in 6cm dishes at a density of $3x10^5$. Cells were treated with Cisplatin, VP, GSK-126, or SAHA or combinatorial treatment for 24h. Cells were collected and washed with 1X PBS. Per the previously established protocol, samples were acquired using Cytoflex and Beckmann Coulter. CytExpert software was used to perform the analysis of acquired data. Early and late apoptotic cells were quantified and represented as a fold increase in apoptotic cells through a bar graph.

2.2.8 Cell cycle analysis through flow cytometry:

Propidium iodide binds to DNA in live cells and hence is used to quantify the total content of DNA, based on which we can assign what phase of cell cycle the cellular population is in. Around 3×10^5 cells were plated in 6cm dishes before they were exposed to drug treatment.

Post exposure, cells were harvested and cleared in 1X PBS, centrifuged at 5000 rpm for 10min at 4 °C. Using ice cold 70% ethanol, cells were slowly vortexed and fixed and were stored overnight at 4 °C. Post this, cells were again suspended in 500µl of 1X PBS with 4µl of propidium iodide (PI) and incubated in dark for 10min. Based on PI uptake, total DNA content of cells was quantified. Through Cytoflex Beckmann Coulter, data was acquired and analysed with CytExpert software.

2.2.9 Estimation of specific mitochondria generated ROS (mtROS) via flow cytometry:

Production of superoxides by mitochondria can be assessed using the dye MitoSox red. This dye selectively targets mitochondria, where it is oxidised by superoxide radicals but not by other reactive oxygen species or reactive nitrogen species. This oxidised fluorescent compound is then detected through flow cytometry with a shift from left to right quadrant. Around 3×10^5 cells were plated in 6cm dishes before they were exposed to drug treatment. Post-exposure, cells were incubated with 1ml of 5µM of MitoSox red for 20 minutes. After this, cells were cleared in 1X PBS and harvested by centrifuging at 3000 rpm for 10min at 4 °C. One rinsing with 1X PBS was done in the dark before acquiring the through Cytoflex Beckmann Coulter, CytExpert software was used to analyse the data obtained.

2.2.10 Estimation of mitochondrial membrane potential:

In healthy mitochondria, JC-1 forms aggregates that fluoresce in the red range (~590nm), while in unhealthy mitochondria, JC-1 aggregates are not formed, and JC-1 moiety emits in the green range (~529nm). 1×10^5 cells were plated in 6cm dishes and were treated with Cisplatin for 24h. JC-1 dye was added to each well at a working concentration of 70nM and incubated for 30min at 37°C, post 24h. Cells were collected and washed with PBS twice. Finally, they were re-suspended in 500µL 1X PBS. Using Cytoflex Beckmann Coulter, sample acquisition was

performed, and acquired data was analysed using CytExpert software. The membrane depolarization is depicted as a red-to-green ratio through a bar diagram.

2.2.11 Immunofluorescence and Phalloidin staining:

With 2.5 × 105 cells/well, HOS cells were seeded on coverslips in 6 well plate. At the desired confluency, cells were treated with the drugs as indicated. Post-treatment, cells were thoroughly washed with 1X PBS and fixed with 2% PFA for 10 min at RT. Post 3 washes with 1X PBS. Cells were permeabilized using 0.1% Triton X-100 for 1 min. 2.5% BSA was used for blocking for 60 minutes. Primary antibody incubation (2.5% BSA; 1:1000) was done at 4 °C overnight. Cells were washed twice with 1X PBS and then incubated with designated Alexa Fluor secondary antibodies (1:2000 in 2.5% BSA) for 60 minutes. Post DAPI incubation for 10mins, coverslips were mounted on slides using 70% glycerol. Coverslips were visualized under Zeiss ApoTome.2 Microscope (Zeiss Axio Observer.Z1/7). Zen2.3 SP1 software was utilized to analyse the images**.**

For F-actin staining through phalloidin, cells were exposed to Phalloidin-iFluor 488 reagent (1:2000 dilution on 2.5 % BSA) for 45 minutes at room temperature. This was followed by multiple 1X PBS washes, and coverslips were mounted on slides using DAPI and imaged under a Zeiss ApoTome.2 microscope.

2.2.12 Co-Immunoprecipitation Assay:

Magnetic beads were washed thrice with 1X PBS-T (1X PBS+0.05% Tween-20). Washed magnetic beads were incubated with anti-YAP antibody (1:50 dilution) for 1h at 4ºC. After three thorough washes with PBS-T, 500-800µg of total protein lysate was added and incubated overnight at 4ºC. This was followed by removing supernatant after magnetization, and 1X Laemmelli buffer was added accordingly. The samples were then heated at 70ºC to release the

protein bound to the antibody. Protein samples were run on SDS-PAGE and immunoblotted as described previously with YAP and EZH2. IgG was used as a negative control, while input samples were used for normalisation.

2.2.13 ChIP-qPCR:

A ChIP assay was performed using MAGnify Immunoprecipitation System (49-2024, Invitrogen). OS cells were seeded in 10cm dishes and were subjected to drug treatment as indicated. Treated cells were collected and washed with 1X PBS three times. Cross-linking was done using 1% formaldehyde. Afterwards, crosslinking was quenched using 1.25M Glycine. Cells were washed with 1X PBS and resuspended in lysis buffer supplemented with protease inhibitor cocktail (50µL per 10^6 cells). After incubating on ice for 10min, cells were vortexed briefly. Using pulse sonicator, the samples were sheared for 60 cycles (45secs ON, 15sec OFF); sheared chromatin was run on 1.2% Agarose gel to visualize shearing. Sheared chromatin was then immunoprecipitated with antibody directed against H3K27me3 at a concentration of 1:50. Immunoprecipitated samples were washed and reversed cross-linked. To obtain purified DNA fragments, proteinase K treatment was done. ChIP-qPCR was performed using primers targeted to amplify regions of human LATS1, YAP, CCNA2 gene promoters.

2.2.14 Luciferase assay

The luciferase reporter allows us to study the transcriptional activity of a gene. The regulatory region of the gene of interest (here, YAP) is cloned upstream of the luciferase gene. If the desired gene can activate the transcription of Luciferin, then the amount of luminescence detected would be proportional to the activity of that particular gene. Around 2.5×10^5 cells/well were seeded in 6 well plates. Cells were then transfected with 8XGTIIC

vector for YAP using Lipofectamine reagent. After 4-6h of transfection incubation, drug treatment was done as indicated. Per the manufacturer's protocol, cells were washed with PBS and then scraped using 1X Lysis buffer provided with the Promega Kit. Post lysis, cells were centrifuged at 2500 rpm for 15 min. Post this, an equal volume of cell lysate was taken 100µl of Luciferase Assay Reagent substrate was added, and the Luminescence reading was immediately taken in GloMax 20/20 luminometer.

2.2.15 siRNA/shRNA mediated knockdown, HDAC and EZH2 inhibition studies:

Cells were transiently transfected with YAP (40nM) siRNA or pBABE-YAP1 plasmid (2 µg) using Lipofectamine 3000 for 6 h, followed by adding drugs for 24h. For HDAC inhibition, cells were treated with 15 µM of SAHA 2 hours before CDDP treatment. For pharmacological inhibition of YAP, HOS cells were treated with 10µM of Verteporfin two hours prior to Cisplatin exposure. Similarly, for EZH2 inhibition, cells were treated with GSK-126 two hours prior to Cisplatin exposure.

2.2.16 Transcriptomic sequencing and *In-silico* analysis:

The total RNA was isolated and taken for fragmentation and priming for transcriptomic sequencing. As per the manufacturer's protocol for NEBNext Poly (A) mRNA magnetic isolation module (Catalog: E7490, New England Biolabs), 500ng of Total RNA was used to enrich the mRNA. NEBNext® UltraTM II RNA Library Prep Kit for Illumina (Catalog: E7775S, New England Biolabs) was utilised to prepare the libraries after the enrichment of mRNA. To being, the mRNAs were primed with random primers from NEBNext before being chemically fragmented in magnesium-based buffer at 94°C for 6 minutes. This was carried out to obtain fragments of ~200 nucleotides. These fragmented mRNAs were reverse transcribed to form cDNA. Thereafter, first-strand cDNA reactions were carried out to obtain double

stranded DNA. 8X of AMPure XP beads (Catalog: A63881, Beckman Coulter) was used to clear the dsDNA fragments. The blunt-ended fragments were adenylated, thereafter the loop adapters were ligated and cleaved to the adenylated fragments with uracil-specific excision reagent (USER) enzyme. Using NEBNext Ultra II Q5 master mix, and "NEBNext® Multiplex Oligos for Illumina, the cDNA was amplified for 8 cycles of PCR. **Adapter sequences:**

P7 adapter read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

The library concentration was determined in a Qubit.3 Fluorometer (Catalog: Q33216, Life technologies using The Qubit dsDNA HS (High Sensitivity) Assay Kit (Catalog: Q32854, ThermoFisher Scientific). The library quality assessment was done using Agilent D5000 ScreenTape System (Catalog: 5067-5588, Agilent) and Agilent D1000 ScreenTape System (Catalog: 5067- 5582, Agilent) in a 4150 TapeStation System (Catalog: G2992AA, Agilent) which is designed for analyzing DNA molecules from 100 to 5000bp. 1µl of the purified library was mixed with 10µl of D5000 sample buffer (Catalog: 5067-5589) and vortexed using IKA vortexer at 2000rpm for 1min and spun down to collect the sample to the bottom of the strip. The strip was then loaded on the Agilent 4150 TapeStation system.

Data quality was checked using FastQC and MultiQC software for base call quality Distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination. Adapter sequences and low base reads were processed using fastp. Transcripts were quantified using Cufflinks, and DESeq2 package was utilised for Differential expression analysis. Transcripts with log2fold change of 1.5 and above were considered as upregulated, and those below -1.5 as downregulated. RT-PCR was carried out to validate the expression of some of the key genes.

2.2.17 Statistical Analysis:

For statistical analysis, we opted for GraphPad Prism software v8.0. Wherever applicable, t-test or one-way ANOVA was utilized to quantify the effect of specific treatment compared to control. When multiple comparisons were analyzed, Tukey's method was used. p-value has been designated on each graph; significant: $p \leq 0.05$ illustrated by symbols * or # or & or @; p-value ≤ 0.01 then denoted by ** / ## /&&/@@; if p-value ≤ 0.001 then depicted by *** / ### /&&&/@@@

S.No	**Instrument Name**	**Manufacturer**
1.	CO_2 incubator	Thermofisher
2.	Laminar airflow	MAC
3.	Vertical Gel Electrophoresis	Biorad
4.	Semi-dry transfer apparatus	Biorad
5.	Flow cytometer	Beckman Coulter
6.	Microplate reader (MultiSkan Sky)	Thermofisher
7.	Real time Thermocycler	Applied Biosystems and Biorad
8.	Confocal Microscope	Zeiss
9.	Apotome Microscope	Zeiss
10.	Inverted Microscope	Olympus

Table 2.4 List of major instruments used in the study

Chapter 3:
Results & Discussion

3.1 Introduction:

Osteosarcoma (OS) is one of the prevalent types of pediatric primary malignant bone tumour, primarily originating from osteoid-producing mesenchymal cells. It accounts for approximately 2.4% of pediatric cancers, putting it at the 8th position in the list of childhood malignancies. OS is extremely heterogeneous, from histological variation to the tumour's location within the bone. Even with advancements in intensive medical research, 40% of OS patients succumb to the disease[22]. The fact that young boys are 1.5 times more likely than young girls to get OS is one of the important aspects of its epidemiology and given that most incidences occur in young people under the age of 20, this malignancy is considered rare. Since the mid-70s, improvements in chemotherapy, including high-dose methotrexate, cisplatin, doxorubicin, and ifosfamide, along with surgical interventions have increased the overall five-year survival rate to 70-80%[12, 14, 16]. While this is promising, newer avenues are required to even further the survival rates of OS patients since it affects largely the teenage population.

Recently, the scientific diaspora has been more focused on developing combinatorial therapies for treating cancers as they have been found to be more effective than the mono-therapy approach. The idea that different drugs potentially target different pathways in an additive or synergistic manner is promising, and this approach can circumvent the inherent issue of drug resistance, inadvertently leading to relapse. Although monotherapy is still largely used, especially in low-income countries[29], it is often the higher toxicity rates that also cause a patient to develop other co-morbidities. With combinatorial therapy in place, the individual dosage of each drug is reduced in order to have a more pronounced effect on cancerous cells and not healthy ones. Separation, strategic dosing and the sequence of administration of each drug defines the landscape of tumour reduction. Inhibitors for ubiquitous responses such as apoptosis, increase in oxidative stress etc. have been tried well with platinum-based drugs in combination; for example, Resveratrol displays chemo-preventive effects via activating GSH

and NRF2, and has shown promising results in breast cancer cells[147]. The idea is to select a pathway and/or gene with differential regulation in normal versus cancerous cells. In this regard, epigenetics is now being hailed as one of the most prominent areas for developing drugs that are actively targeting chromatin-modifying enzymes. Mutations in the chromatin-modifying enzymes or their aberrant activities can potentially drive a tumour microenvironment[9].

Histone deacetylase and DNA methyltransferase inhibitors have been extensively studied and clinically tried in the last decade[77]. HDACs exhibit several anti-cancer effects such as cell cycle arrest, apoptosis, and inhibition of angiogenesis, which makes it a perfect candidate. The first approved HDAC inhibitor was Vorinostat or SAHA, also known as a pan-inhibitor for solid malignancies, especially T-cell lymphoma. Similarly, DNMT inhibitors also are being evaluated for their efficacy now. Usually, in cancers, the promoter regions of tumour suppressor genes are hypermethylated making them inactive; hence, DNA hypomethylating enzyme inhibitors were discovered. The most commonly used DNMT inhibitors are 5-azacytidine and its derivative, 5-2'-deoxycytdine, while recently, more inhibitors, such as MG98, hydralazine amongst a few others have been investigated for their potency.

Studies have individually illustrated the effects of these chromatin modifying enzymes, however how they function in combination with anti-tumour drugs still has not been completely elucidated. The reversibility of chromatin landscape[47] is the biggest window of opportunity to design new therapy modules. In lieu of this, understanding the molecular aspects of how these combinatorial strategies may work, especially the pathway is crucial. One such signalling pathway is Hippo pathway that is often dysregulated in many cancers. Yes-associated protein (YAP), a downstream effector of this pathway, is a transcriptional coactivator that undergoes a phosphorylation/de-phosphorylation cycle by the Hippo signaling cascade[108]. When Hippo signaling is impaired, phosphorylation of YAP is reduced, shuttling it to the nucleus; once

inside the nucleus, YAP is involved in many cellular homeostasis processes such as cell proliferation, survival, and growth. There is now increasing evidence that elevated expression of YAP/TAZ is associated with poor prognosis in many cancers. Concerning pediatric bone cancers like OS, YAP may be involved in multiple ways- from angiogenesis to metastatic dissemination. Interestingly, YAP, a key player in developmental programs, is also effectively involved in mediating certain chromatin changes, such as associating with NuRD complexes[146]. However, its precise role in the regulation of epigenome is considerably understudied.

In the current study, we begin with analysing the chromatin landscape post drug exposure to OS cells. We further establish crosstalk between signalling pathway (YAP) and chromatin modifier (EZH2), understanding their dynamics, and finally, we establish how we can sensitize the OS cells based on our findings to potentially work as a combinatorial therapeutic strategy with cisplatin.

3.2 Results:

3.2.1 Comparative analysis of cells exposed to differential doses of CDDP

Viability for HOS cells was determined at 24h for different doses of CDDP (**Fig.3.2.1a).** HOS cells were given a short CDDP (3.3mM, CDDP Challenge) pulse treatment for 2h. While most of the population died, few cells that survived were taken for total RNA processing. Based on the effective dose used in clinical applications, in-vitro treatment dose for CDDP treatment was determined[148]. Deep mRNA sequencing was performed for cells treated, and transcriptomic analysis was carried out using DESeq2 package. In our sequencing analysis, we observed more differentially downregulated transcripts than upregulated transcripts, suggesting an overall shutdown of transcriptional machinery **(Fig.3.2.1b**). The mRNA expression pattern obtained

from our samples was further compared and correlated to patient transcriptomic data obtained from GEO database (***Accession Number: GSE99671***). Based on the Log2fold cutoff of 1 and p-value ≤0.05, we segregated the transcripts as upregulated and downregulated. Of the differentially regulated transcripts, 82 transcripts were unique to control, while 865 were unique to treatment. Around 3.8% of transcripts were common between the OS cell line and patient data (**Fig.3.2.1c)**. As we were interested in histone modifications per se, we segregated four different classes of histone modifiers, namely: Histone Deacetylases (HDACs), Histone Demethylases (HDMs), Histone Acetyl Transferases (HATs), and Histone Methylases (HMTs) (**Fig.3.2.1d, 3.2.1e, 3.2.1f, 3.2.1g**). Further, we did a comparative analysis between differentially regulated chromatin regulators and patient data to find the common chromatin modulators between the two sets; we found a total of 8 transcripts (1%) common between cell line and patient data (**Fig.3.2.1h)**. We further carried out gene ontology analysis for the common genes and segregated them into the following GO processes: Molecular Function, Biological Process, and Cellular Component (**Fig.3.2.1i, 3.2.1j, 3.2.1k**). Based on our GO analysis, we concluded that apart from chromatin regulatory processes, chromatin modulators were also involved in the cell cycle process. Further, we performed a Cytoscape analysis to look for interacting pathway network (**Fig. 3.2.1l, 3.2.1m, 3.2.1n, 3.2.1o, 3.2.1p)** where we observed major pathway such as TP53, Notch signalling getting regulated by chromatin modifiers along-with pathways involved in mitochondrial biogenesis, DNA damage repair amongst others. Finally, key chromatin related genes getting dysregulated after CDDP treatment were identified by utilizing protein–protein interaction (PPI) network and CytoHubba plugin of Cytoscape. MCC algorithm was used and we found HDACs and HMTs functionally working together as key genes (**Fig.3.2.1q).**

To validate our sequencing data, we performed real-time PCR of the selected HDACs (**Fig.3.2.1r)**. In corroboration to our transcriptomic data, we found an increase in both HDAC5

and HDAC9 after the CDDP challenge. Conventional chemotherapeutics are usually administered at a maximally tolerated dose or generally a high dose for 3-4 cycles with alternate drug-free period to allow patients to recover from adverse drug effects. Despite its efficacy, recurrence is still at loom. Hence, research is now directed toward using a low or metronomic dose of chemotherapeutic drugs to allow for dose adjustments and enhanced adjuvant sensitization. In this regard, a study conducted in 2015 found positive results in nasopharyngeal and uterine cervical cancers when given prolonged low-dose therapy[149]. However, most studies have been empirical till now, with little or no studies defining the molecular features of tumour cells exposed to sub-lethal doses of anti-cancer drugs.

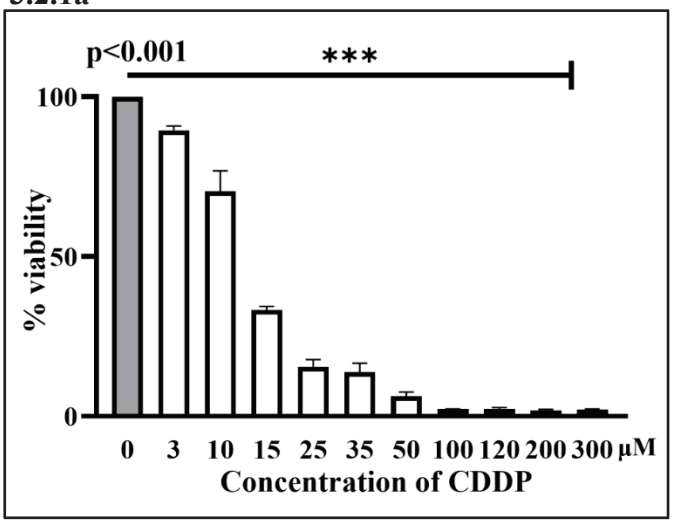
Taking cues from the literature review, we decided to opt for a sub-lethal of low dose for our further study. Using our cellular viability data (**Fig.3.2.1a)**, we first validated the expression of HDAC5 and HDAC9 in human OS cells treated with a 3µM (***'sub-toxic'***) dose. We found their expression consistently increasing, suggesting that HDACs are highly expressed in cancer cells and, upon treatment with a CDDP, are elevated further (**Fig.3.2.1s**). Since we observed a dynamic patterning of these chromatin-modifying enzymes, especially upregulated histone deacetylase and a simultaneous transcriptional shutdown, we were then intrigued to characterise this repressive state post drug exposure. Intriguingly, at both the CDDP challenge (3.3mM) and CDDP (3µM), we saw an increase in the levels of both the repressive histone marks: H3K27me3 and H3K9me3; however, we saw a more robust change in the expression of H3K27me3, a mark of facultative chromatin (**Fig.3.2.1t, 3.2.1u**). Herein, since we observed bleed through in immunoblots, we have used two different loading controls - GAPDH and Total H3.

The role of histone, 3 lysine 27 tri-methylation (H3K27me3) in maintaining proper differentiation throughout development is well established. Likewise, aberrant expression of H3K27me3 is also involved in poor prognoses and therapy evasion[150, 151]. As we observed

a more consistent change in H3K27me3, we moved ahead with this modification for our further studies**.** Through immunofluorescence, we show that H3K27me3 appealingly localises around the nuclear periphery post-CDDP exposure and is present inside the nucleus (**Fig.3.2.1v**). This suggests that changes in global levels of H3K27me3 are correlated to chemotherapeutic insult, even at its sub-toxic dose.

We next checked the expression of the "writer" of H3K27me3, enhancer of zeste 2 (EZH2). We looked at the expression of EZH2 increasing at both transcriptional and translational levels (**Fig.3.2.1w**). EZH2 primarily localizes in the nucleus, and herein, through immunofluorescence, we show a marked increase in the nuclear localization of EZH2 post-CDDP treatment **(Fig.3.2.1x).**

3.2.1a



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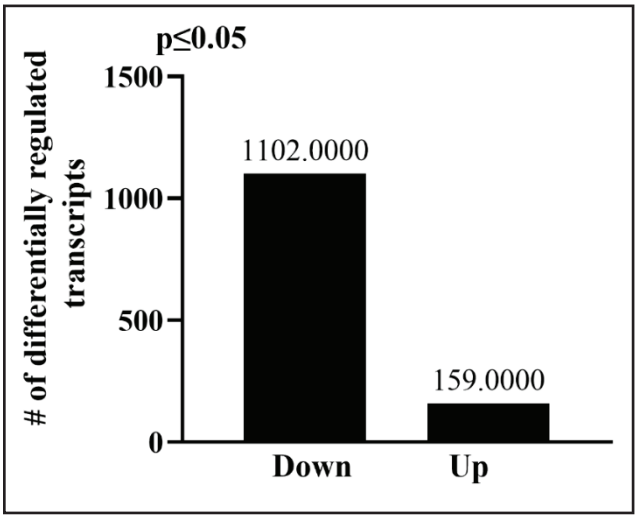

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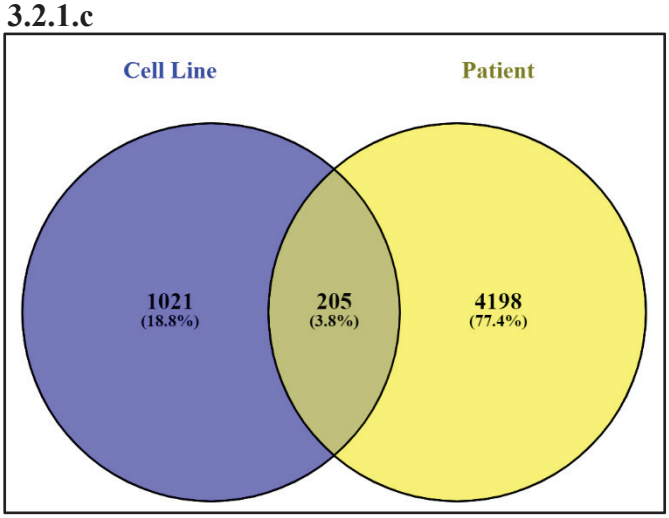

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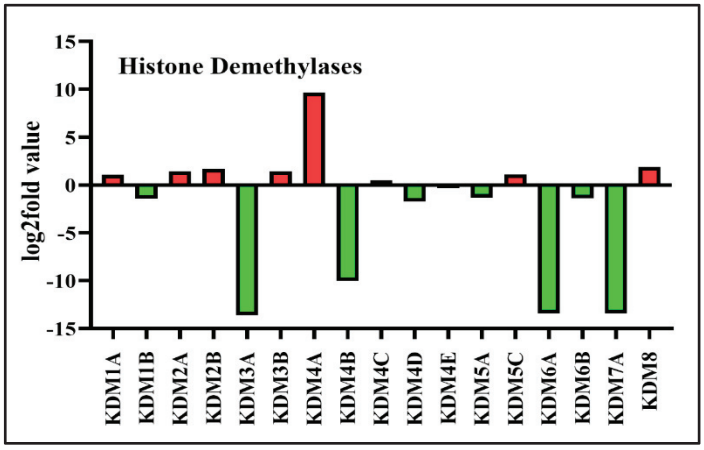

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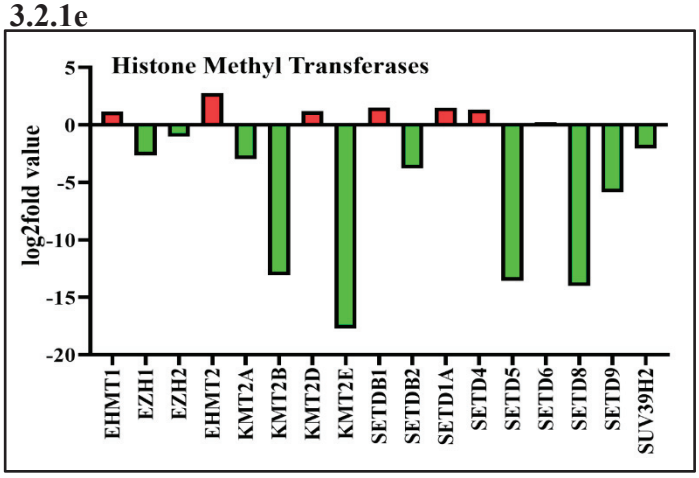

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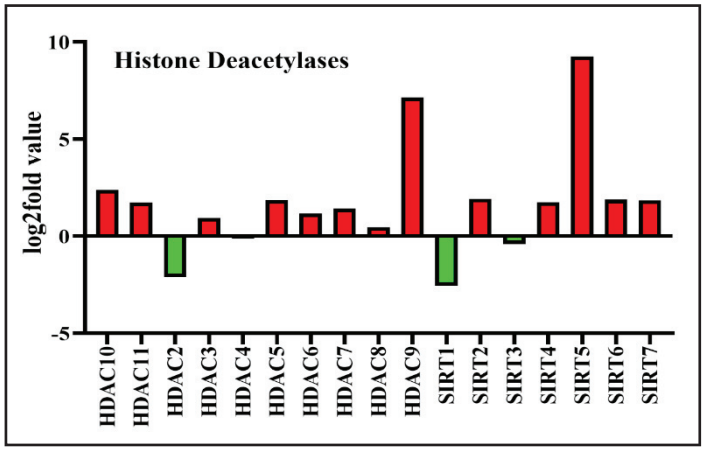

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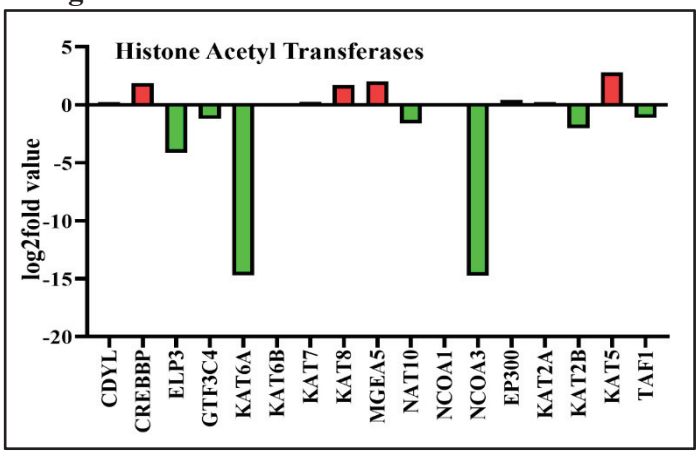

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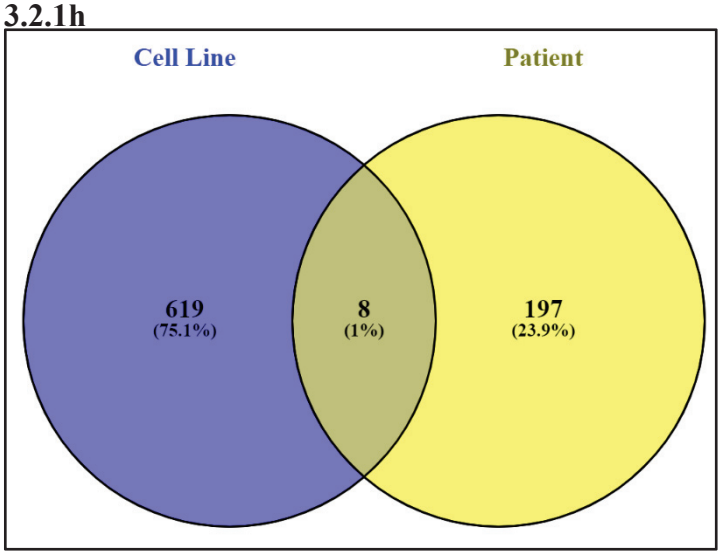

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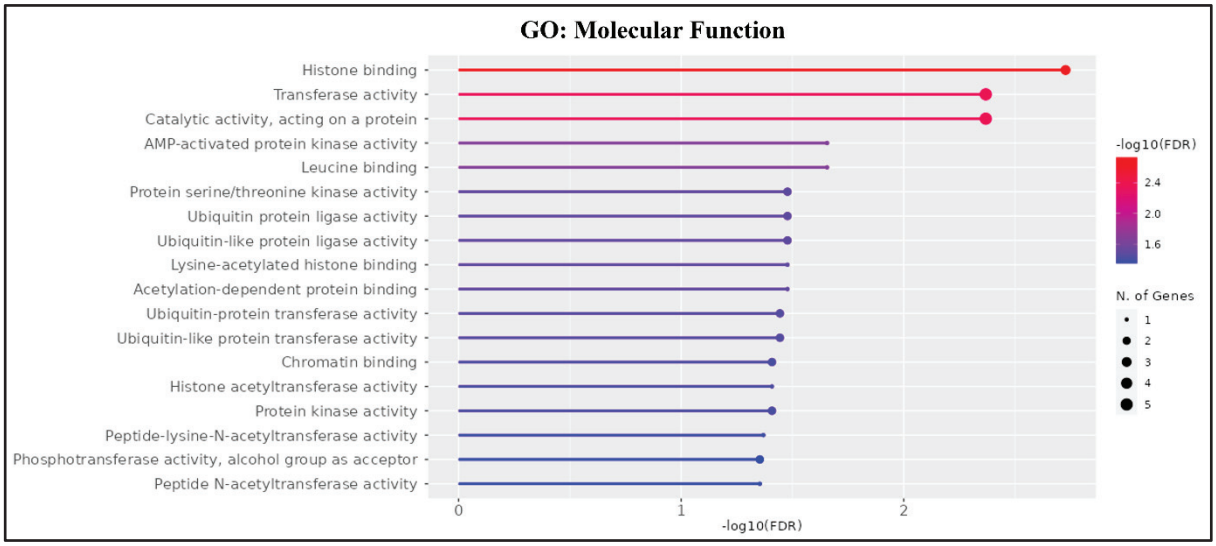

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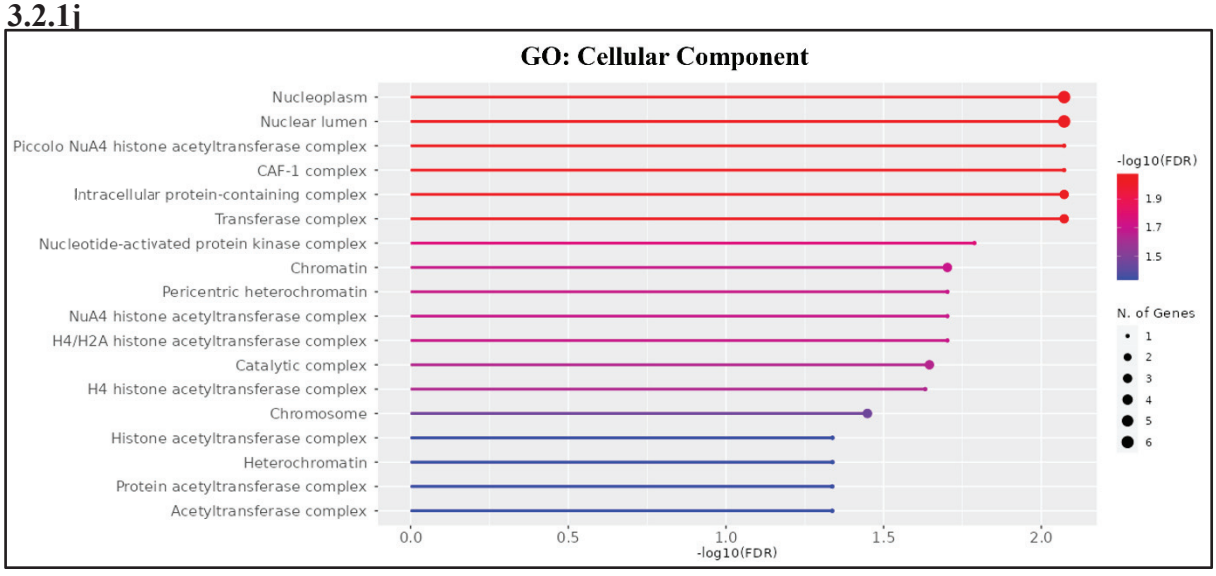

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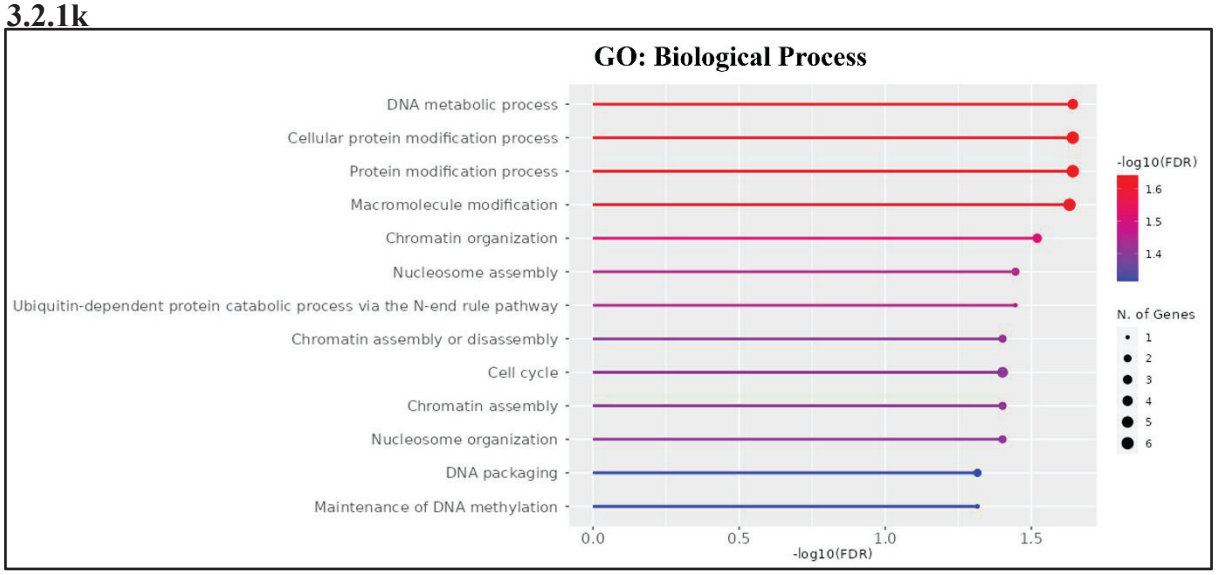

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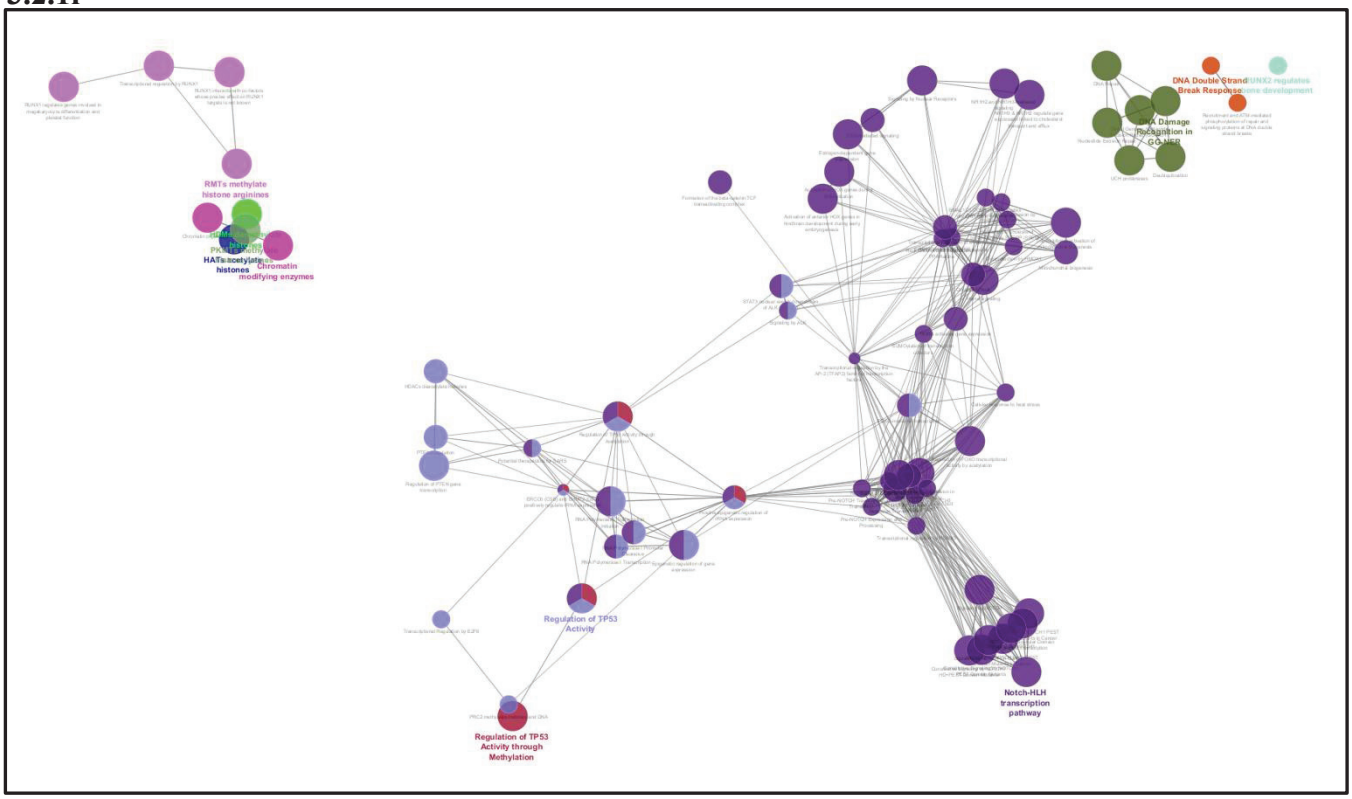
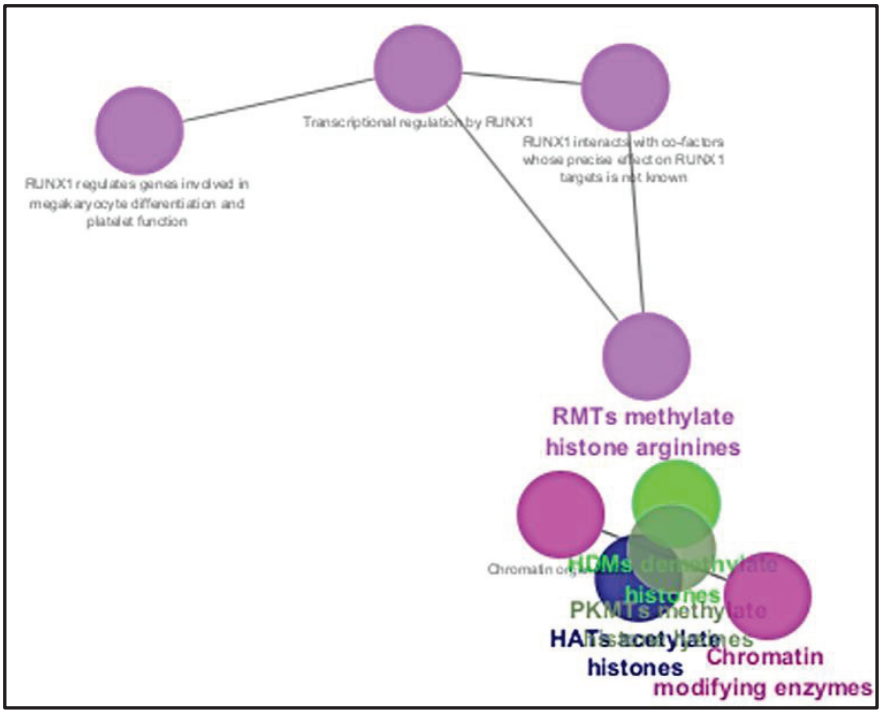
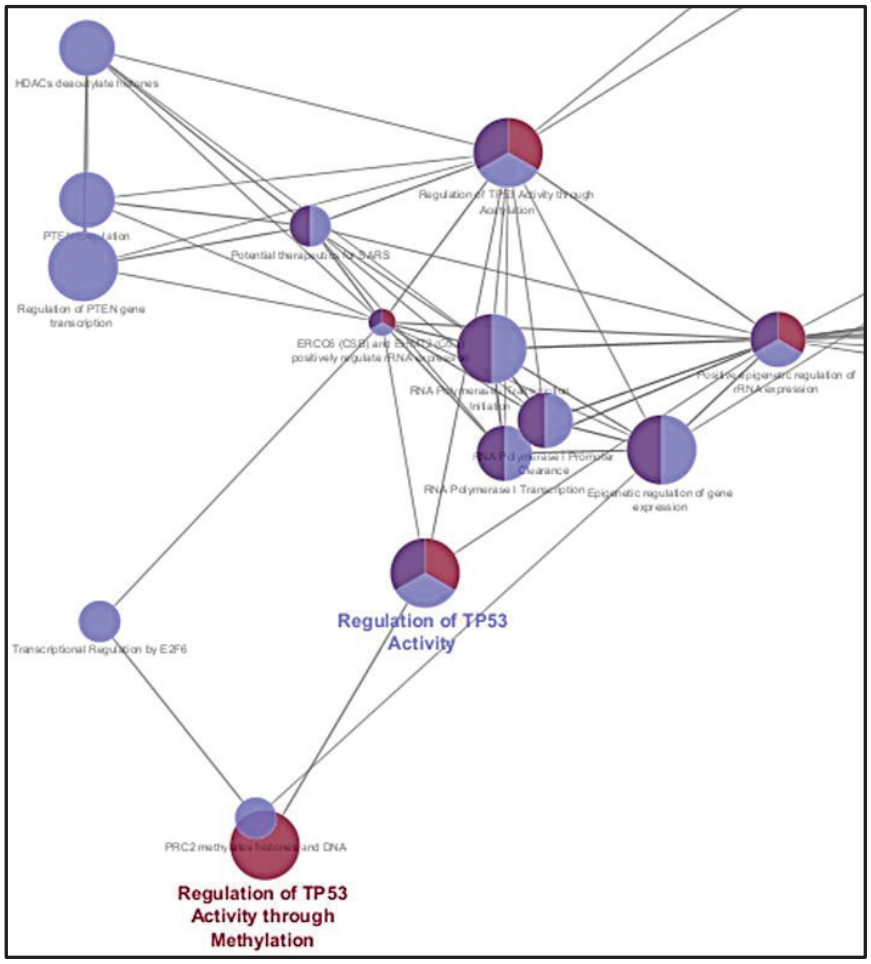

RMTs methylate
histone arginines
HATs acetylate
histones
Chromatin
modifying enzymes
DNA Double Strand
Break Response
Regulation of TP53
Activity
Regulation of TP53
Activity through
Methylation
Notch-HLH
transcription
pathway

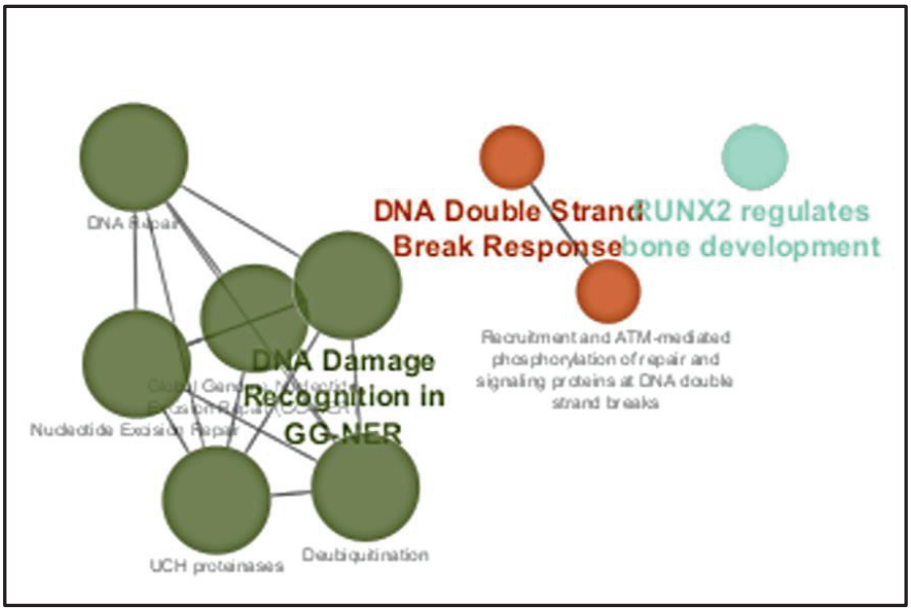
3.2.1m


Transcriptional regulation by RUNX1
RUNX1 regulates genes involved in
megakaryocyte differentiation and
platelet function
RUNX1 interacts with co-factors
whose precise effect on RUNX1
targets is not known
RMTs methylate
histone arginines
HDMs demethylate
histones
PKMTs methylate
histones
HATs acetylate
histones
Chromatin
modifying enzymes

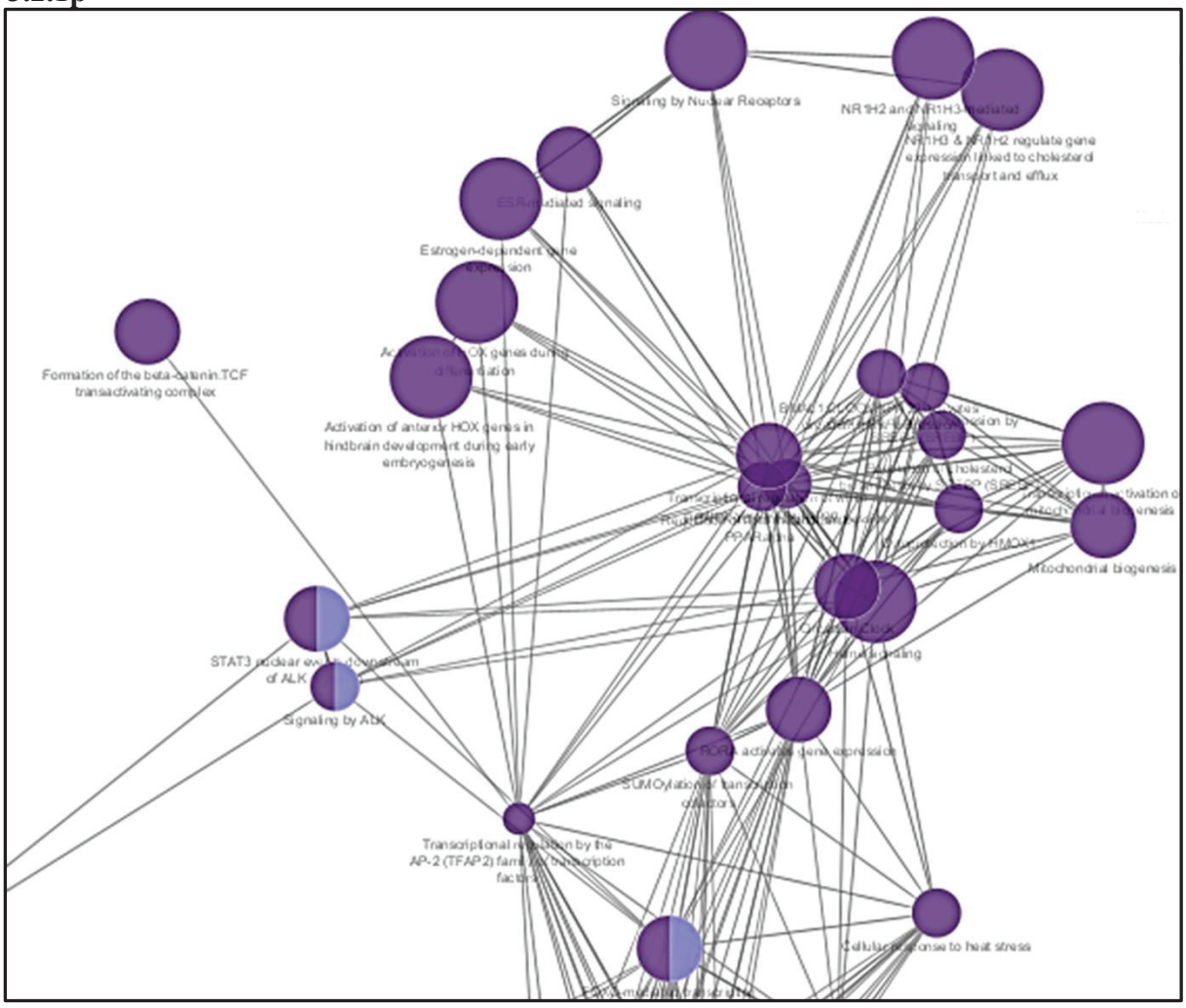
3.2.1n


HDACs deacetylate histones
Regulation of TP53 Activity through Acetylation
PTEN regulation
Potential therapeutics for SMARS
Regulation of PTEN gene transcription
ERCC6 (CSB) and EHMT2 (G9a) positively regulate rRNA expression
RNA Polymerase I Promoter Opening
RNA Polymerase I Promoter Clearance
RNA Polymerase I Transcription
Epigenetic regulation of gene expression
Post-translational regulation of rRNA expression
Transcriptional Regulation by E2F6
Regulation of TP53 Activity
PRC2 methylates histones and DNA
Regulation of TP53 Activity through Methylation

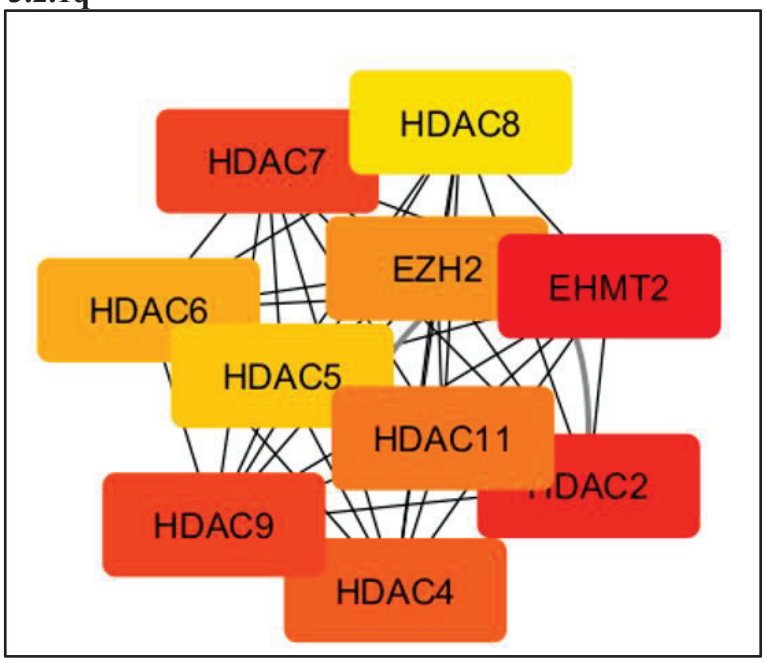
3.2.1o


DNA Repair
DNA Double Strand Break Response
RUNX2 regulates bone development
Recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks
DNA Damage Recognition in GG-NER
Nucleotide Excision Repair
UCH proteinases
Deubiquitination

3.2.1p


Signaling by Nuclear Receptors
NR1H2 and NR1H3-mediated signaling
NR1H3 & NR1H2 regulate gene expression linked to cholesterol transport and efflux
ESR-mediated signaling
Estrogen-dependent gene expression
Formation of the beta-catenin:TCF transactivating complex
Activation of anterior HOX genes in hindbrain development during early embryogenesis
Mitochondrial biogenesis
STAT3 nuclear events downstream of ALK
Signaling by ALK
Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors
Cellular response to heat stress

3.2.1q



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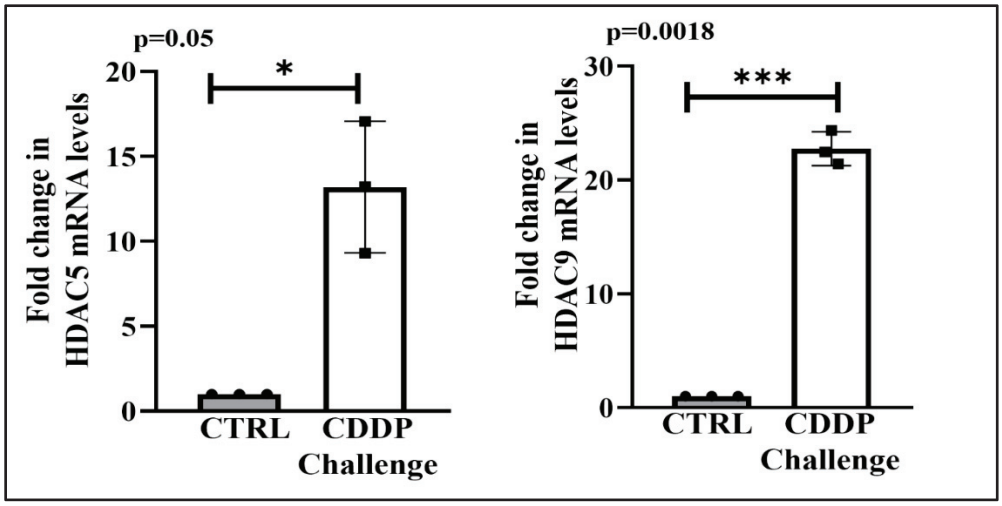

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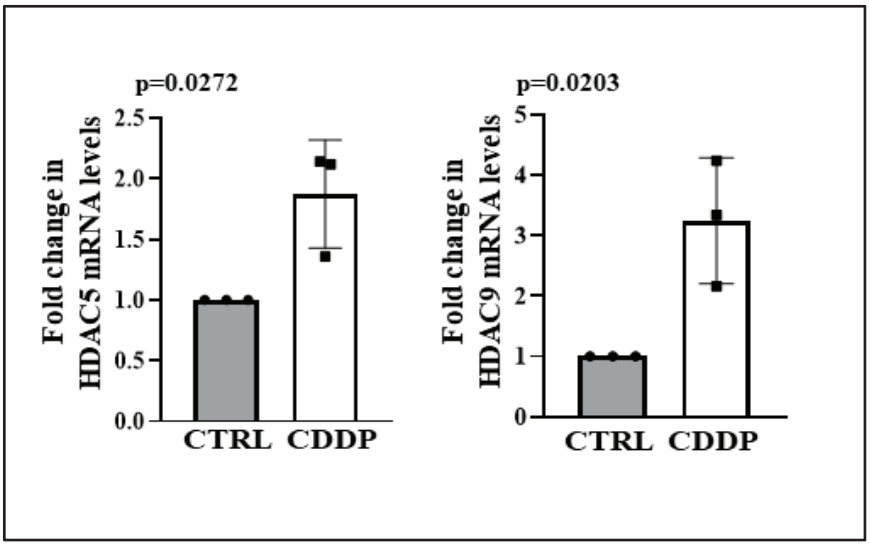

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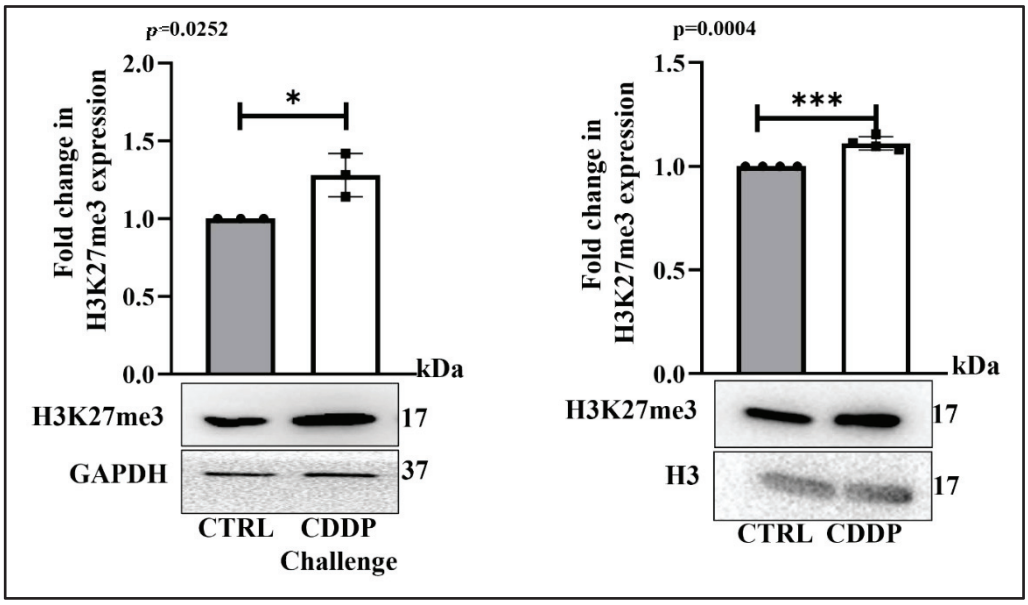
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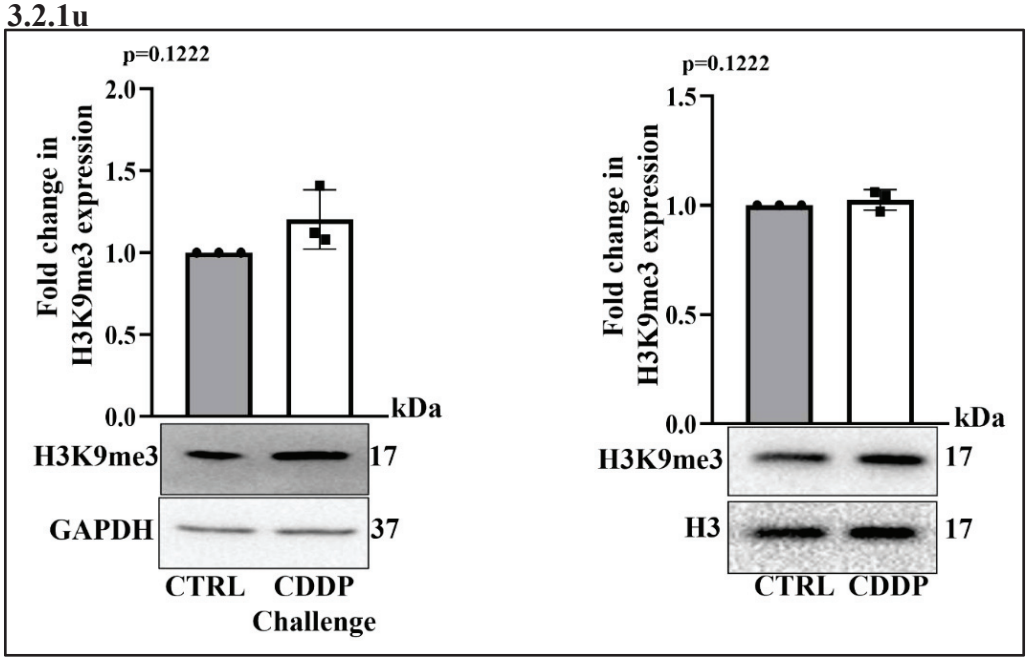
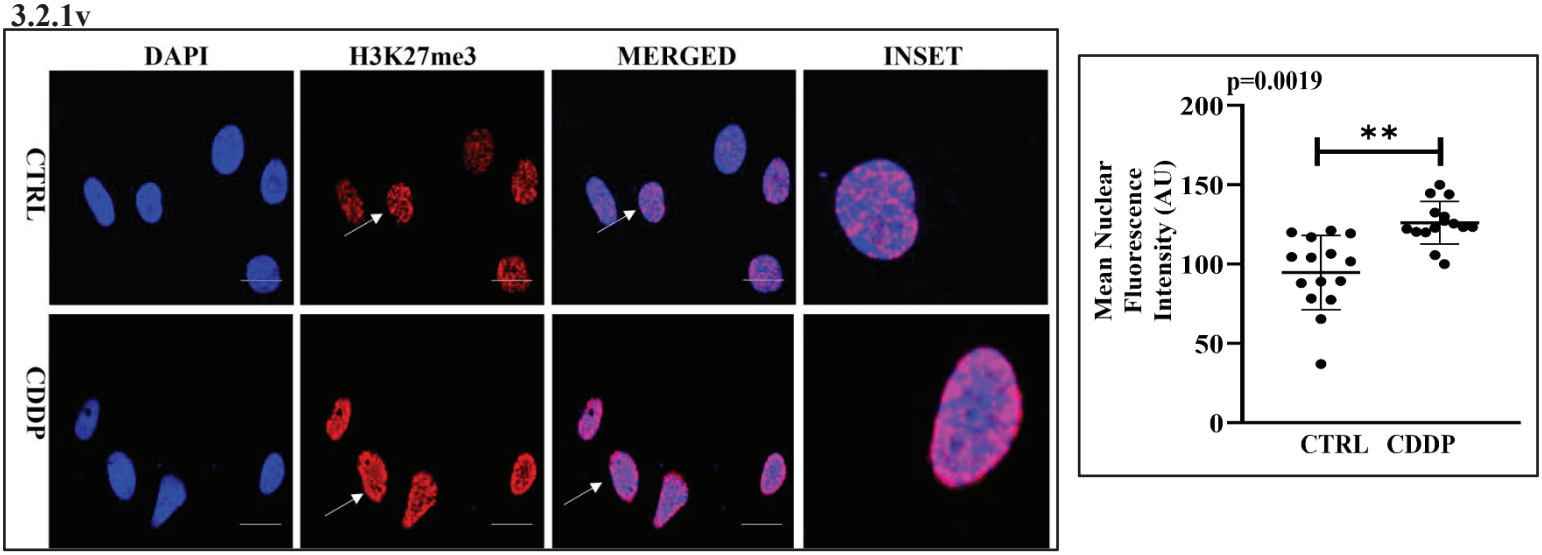
Fold change in H3K27me3 expression
2.0
1.5
1.0
0.5
0.0
*
kDa
H3K27me3
17
GAPDH
37
CTRL
CDDP
Challenge
p=0.0004
Fold change in H3K27me3 expression
1.5
1.0
0.5
0.0
***
kDa
H3K27me3
17
H3
17
CTRL
CDDP

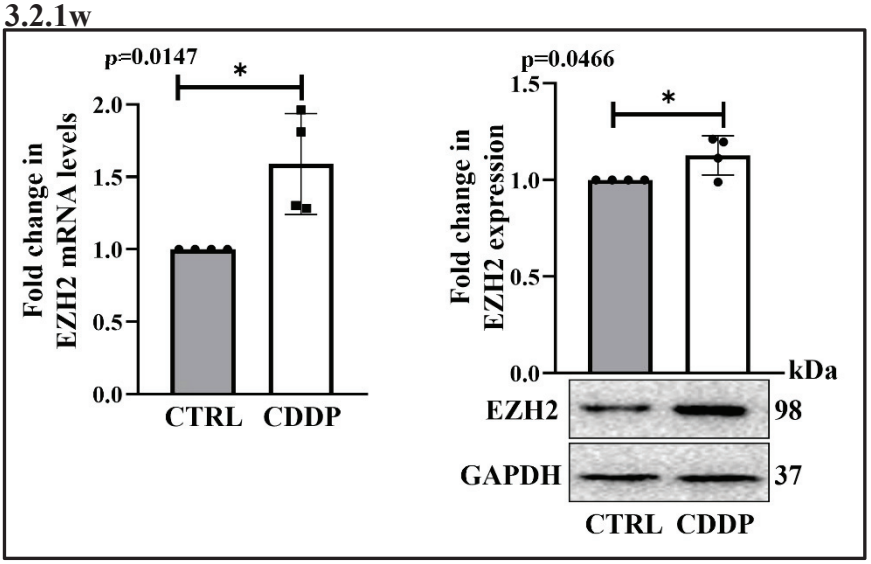
3.2.1u


p=0.1222
Fold change in H3K9me3 expression
2.0
1.5
1.0
0.5
0.0
kDa
H3K9me3
17
GAPDH
37
CTRL
CDDP
Challenge
p=0.1222
Fold change in H3K9me3 expression
1.5
1.0
0.5
0.0
kDa
H3K9me3
17
H3
17
CTRL
CDDP

3.2.1v



3.2.1w



3.2.1x

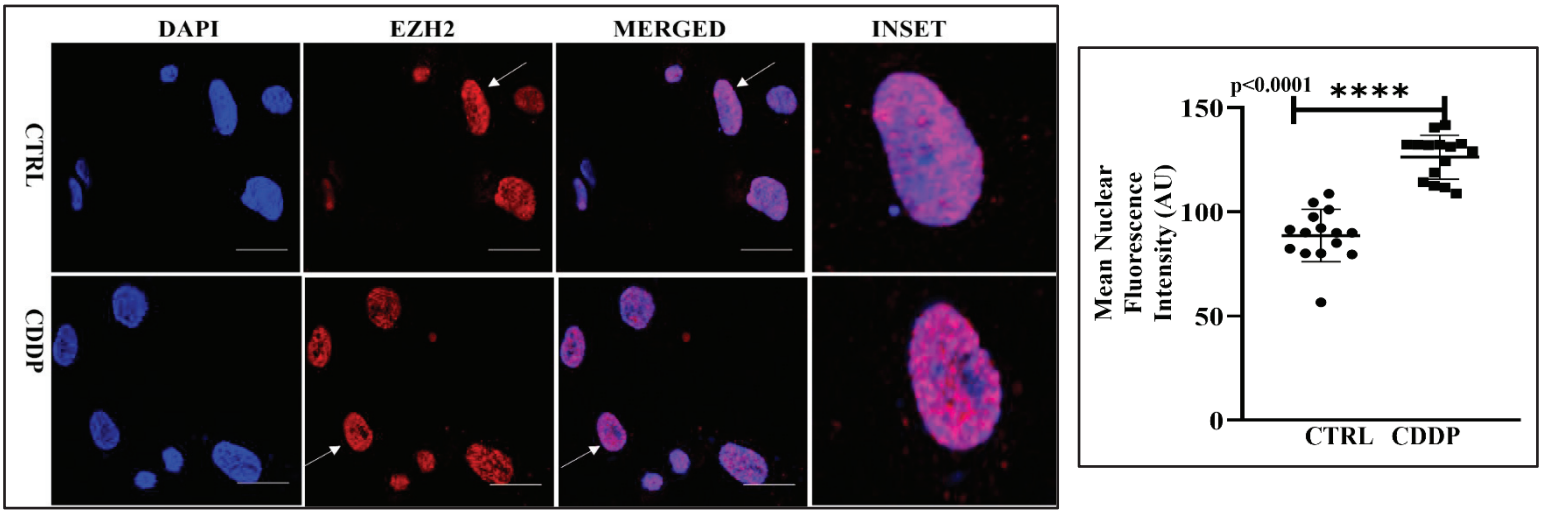


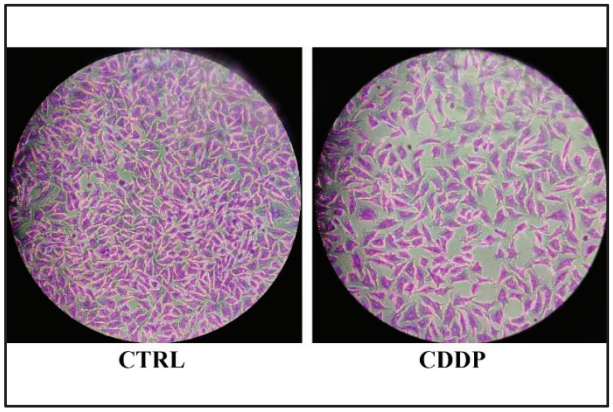
Fig. 3.2.1 Comparative analysis of cells exposed to differential doses of CDDP **a)** Cell Viability (MTT) assay at different concentrations of CDDP for 24h **b)** Number of differentially regulated transcripts, p-value≤0.05 **c)** Venn diagram showing comparative transcripts between transcriptomic data and patient data **d, f, e, g)** Bar graph showing differential Histone modifiers **h)** Venn diagram showing comparative transcripts of chromatin regulators between transcriptomic data and patient data **i,j,k)** Gene Ontology analysis using ShinyGO v0.75 of the common chromatin modifiers between cell and patient transcriptomic data **l)** Cytoscape network of chromatin modifiers listed**,** showing different pathways that are regulated by the chromatin modifiers **m,n,o,p)** Snippets of Cytoscape network **(3.2.1l)** of chromatin modifiers listed**,** showing different pathways that are regulated by the chromatin modifiers **q)**Key gene analysis using CytoHubba **r)** Validation of HDAC5 and HDAC9 post CDDP challenge dose **s)** Validation of HDAC5 and HDAC9 post CDDP treatment for 24h **t)** Immunoblot showing the expression of H3K27me3 post-CDDP and CDDP challenge dose **u)** Immunoblot showing the expression of H3K9me3 post CDDP and CDDP challenge dose **v)** Immunofluorescence staining of H3K27me3 (scale bar: 20µm, ImageJ) **w)** Bar graph showing mRNA expression of EZH2 and Immunoblot showing the expression of EZH2 post CDDP treatment, respectively **x)** Immunofluorescence staining of EZH2 **(**scale bar: 20µm, ImageJ). All values are represented as mean±SD; n=3. Unpaired t-test was applied with (*) $p<0.05$ to estimate the significance when two groups were compared. CTRL represents untreated cells.

3.2.2 Increase in repressive chromatin marks is associated with growth arrest in OS cells

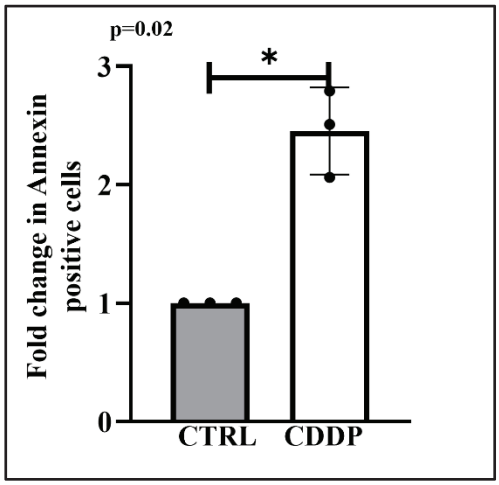
To explore the functional effect of the enriched repressive chromatin marks, we attempted to correlate it to the phenotypic changes we observed post-CDDP exposure. The 'sub-toxic' dose of CDDP understandably inflicted a minimal cellular death shown through crystal violet assay **(Fig.3.2.a)**. This was further confirmed through flow cytometric Annexin PI assay **(Fig.3.2.2b)**. Drug interventions usually disrupt the cell cycle; hereafter CDDP treatment, we found

increased population of cells in Sub-G1 phase, suggestive of cellular stasis/exit from the cell cycle **(Fig.3.2.2c)**. This was confirmed through further experimentations, with a decrease in the expression of proliferative gene, PCNA that is involved in DNA replication **(Fig.3.2.2d)** whereas the expression of CDKN1A/p21- a cyclin-dependent kinase inhibitor was close to being statistically significant (**Fig.3.2.2e)**. To understand if there exists an epigenetic dependency of the non-dividing state, we analyzed enrichment of the histone repressive mark (H3K27me3) at the upstream promoter element of the gene CCNA2 (CyclinA2), which is known to be associated with an active cell cycle state and is also involved in G1/S and G2/M transition. Importantly, in corroboration to our earlier findings, we not only observed H3K27me3 enrichment at the promoter region (amplified segment was within 1kb upstream from the TSS) of CCNA2 through ChIP-qPCR, but the transcript level of the gene also depicted a significantly reduced expression (**Fig.3.2.2f and 3.2.2g)**. Our data thus suggests that gene-specific transcriptional repression associated with drug exposure affects cell cycle gene

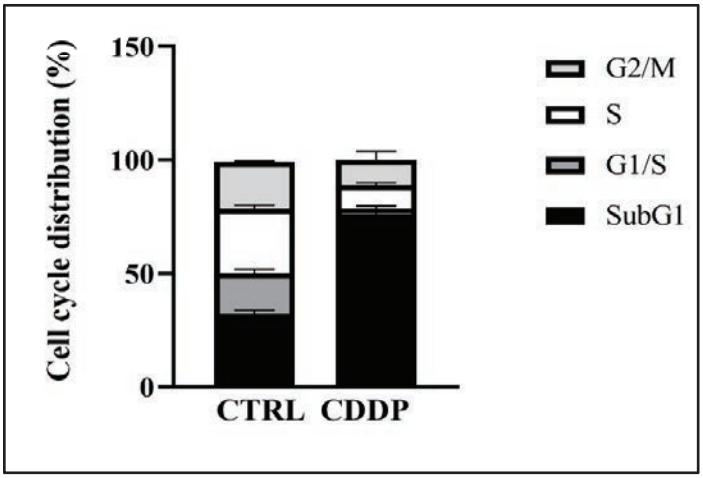
3.2.2a



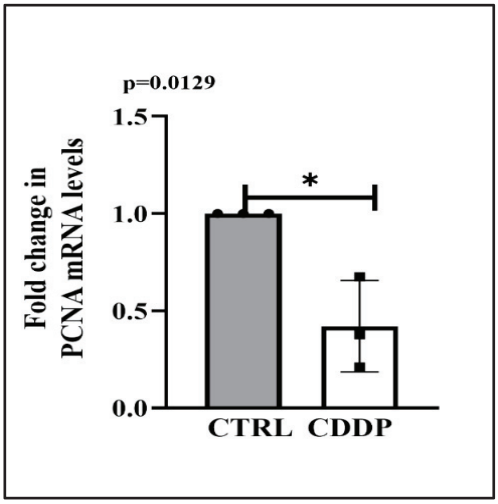
3.2.2b



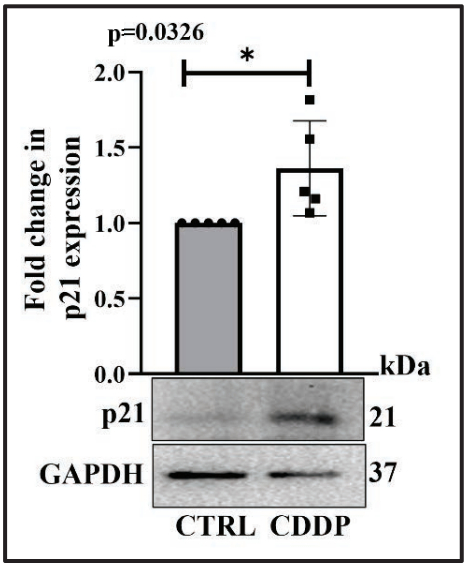
3.2.2c



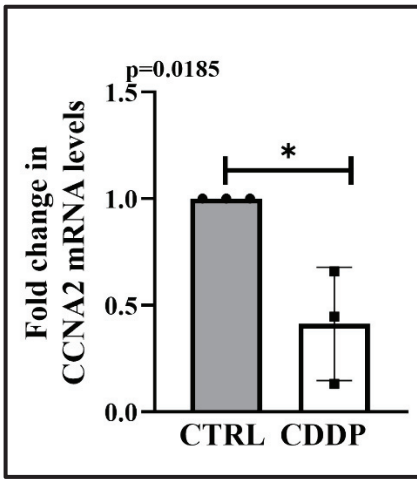
3.2.2d



3.2.2e



3.2.2f



3.2.2g

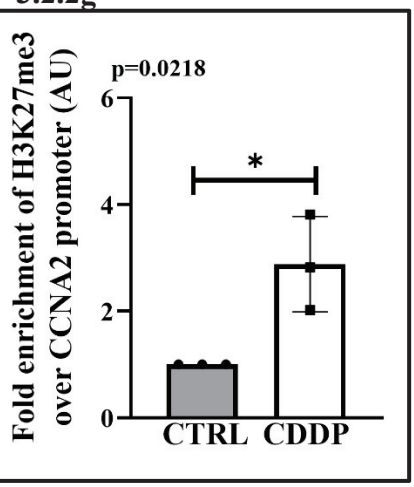


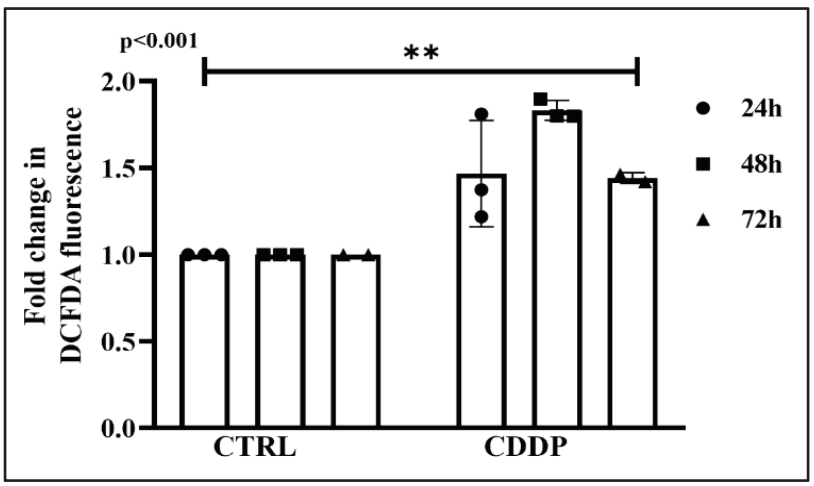
Fig. 3.2.2 Increase in repressive chromatin marks is associated with growth arrest in OS cells a) Crystal Violet Image illustrating cytostatsis post CDDP treatment **b)** Annexin PI analysis post CDDP treatment **c)** Cell cycle analysis through flow cytometry at 24h under CDDP exposure wrt untreated cells **d)** Change in mRNA expression of PCNA at 24h **e)** Immunoblot showing expression of p21 at 24h; GAPDH served as the loading control **f)** Change in mRNA expression of cell cycle gene-CCNA2 at 24h **g)** Enrichment of H3K27me3 over the cell cycle genes (CCNA2) through ChIP-RTPCR**.** All values are represented as mean±SD; n=3. Unpaired t-test was applied with (*) $p<0.05$ to estimate the significance when two groups were compared. CTRL represents untreated cells.

3.2.3 ROS regulate repressive epigenomic marks responsible for growth arrest

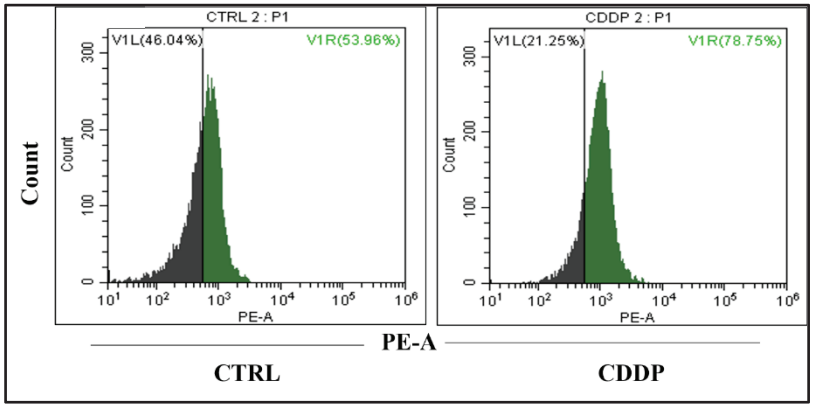
Generally, genotoxic stressors such as anticancer drugs often accompany increased reactive oxygen species (ROS) levels. A tight regulation of the cellular ROS is fundamentally crucial for a cell[152]. In our study, to determine the level of ROS, we performed a DCFDA assay. There was a marked increase in the levels of ROS post-CDDP exposure at all the time points studied (**Fig.3.2.3a)**. Since mitochondria contribute to approximately 90% of cellular ROS, we also looked at the level of mitochondrial ROS **(Fig.3.2.3b)** and found a 1.2-fold increase in its levels post-CDDP exposure. This was further associated with alterations in the mitochondrial membrane potential post CDDP treatment **(Fig.3.2.3c).** Though CDDP generated a high total and/or mitochondrial ROS levels, it was not sufficient to induce distinct cell death and rather more cytostatic effect, as described earlier, was observed. As we previously established the functional role of the enriched repressive marks, we were then interested in understanding if ROS has any role in the modulation of the H3K27me3 mark post-CDDP treatment, as oxidative stress has been implicated in regulating epigenomic alterations. To decipher the association of repressive marks with ROS, we added an antioxidant ROS/quencher: N-acetyl cysteine (NAC) before treating the cells with CDDP and intriguingly found the levels of EZH2 go down vis-à-

vis only CDDP exposure, and concomitant with reduced methyl transferase expression as shown through immunoblot in **Fig.3.2.3d**. We were also interested in understanding if ROS could alter the localization of EZH2, but we found no change in the localization, albeit only reduced intra-nuclear fluorescence intensity of EZH2 after NAC treatment **(Fig.3.2.3e)**. As is with EZH2, decreased expression of H3K27me3 was also observed post NAC treatment (**Fig.3.2.3f)**. In addition, the HOS cells showed reduced cytostatic effect once ROS was quenched **(Fig.3.2.3g)**, which was substantiated by the decreased levels CDKN1A/p21 post NAC treatment as shown in **Fig.3.2.3h**. This prompted us to investigate into whether the change in expression of proliferative marker- CCNA2 after CDDP exposure, as mentioned earlier, is ROS-mediated or not. Interestingly, we found an increased CCNA2 expression with a simultaneous decrease in H3K27me3 enrichment over its promoter upon CDDP plus NAC treatment, suggesting that ROS is the mediator for gene-specific epigenetic modulation after CDDP exposure (**Fig.3.2.3i)**. The above findings show that oxidative stress can regulate histone methylation to modulate genes involved in cellular growth, as shown in the graphical chart **Fig.3.2.3j**.

3.2.3a



3.2.3b



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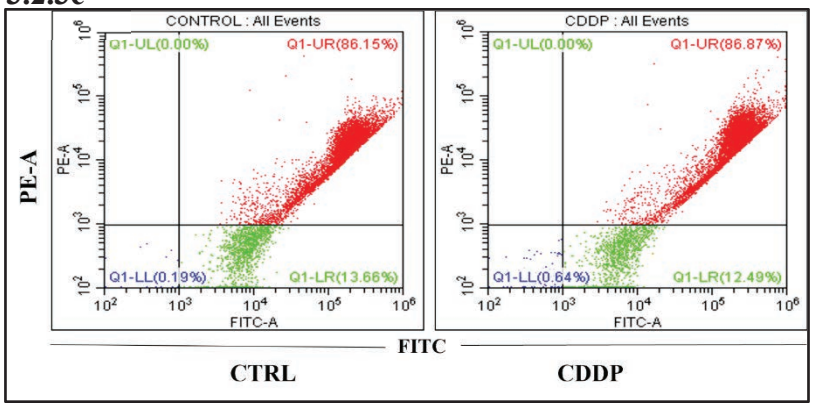
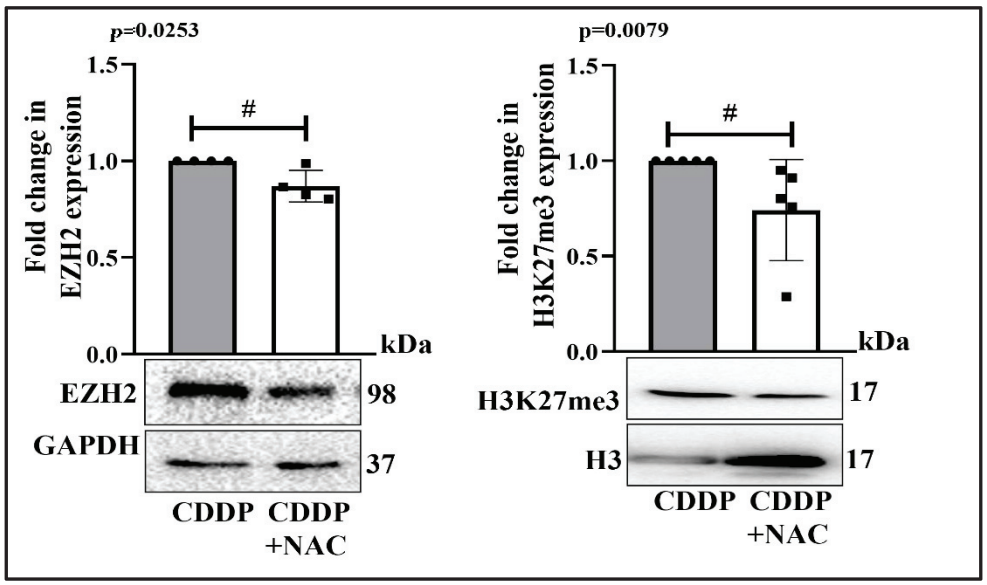
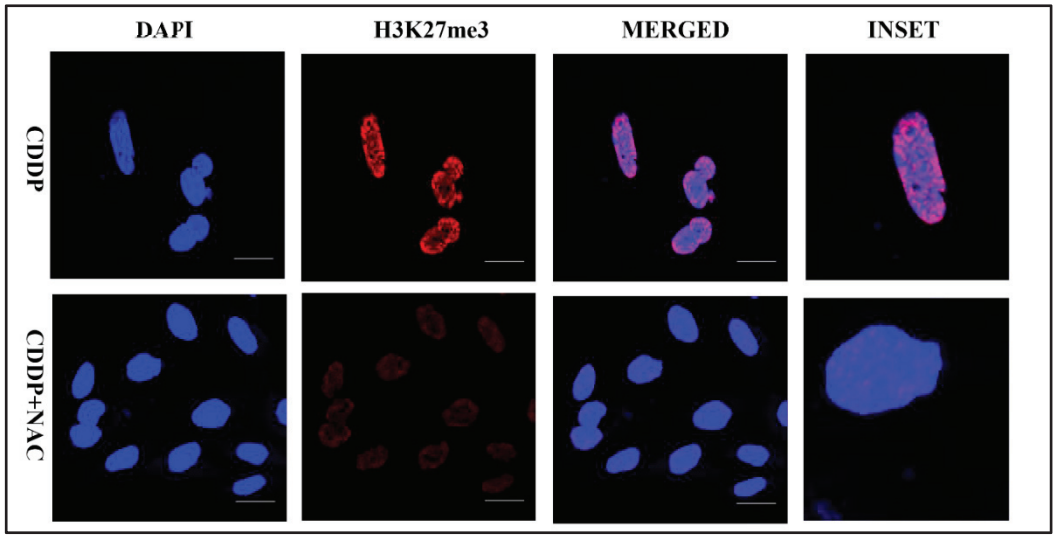
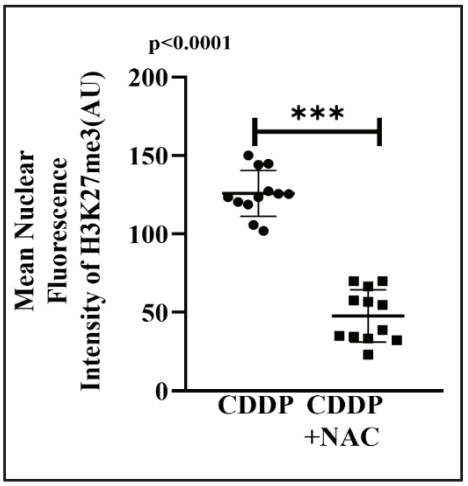


3.2.3d

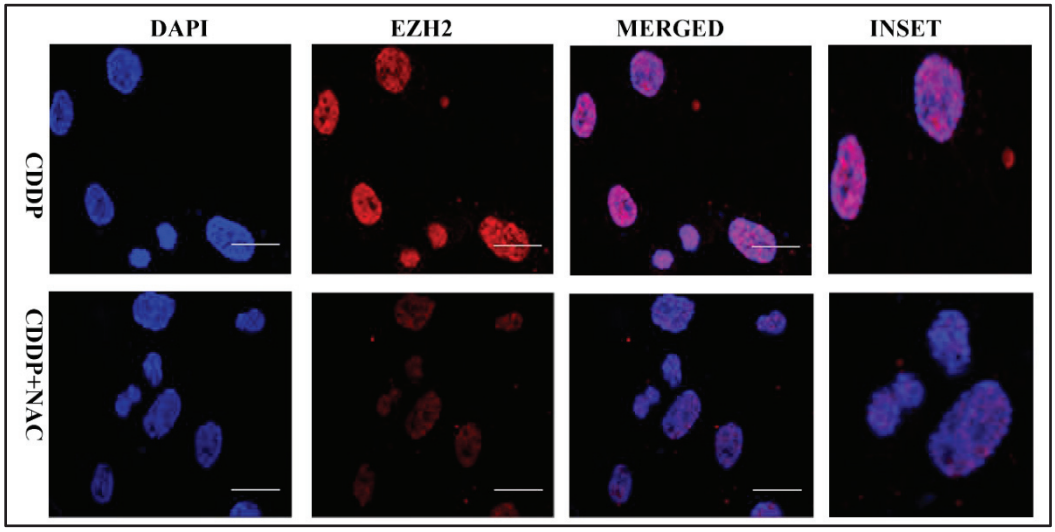


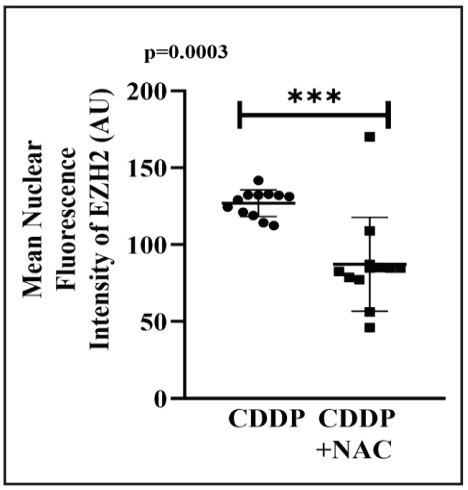
3.2.3e


DAPI
H3K27me3
MERGED
INSET
CDDP
CDDP+NAC


p<0.0001
***
Mean Nuclear Fluorescence Intensity of H3K27me3(AU)
200
150
100
50
0
CDDP
CDDP +NAC

3.2.3f


DAPI
EZH2
MERGED
INSET
CDDP
CDDP+NAC


p=0.0003
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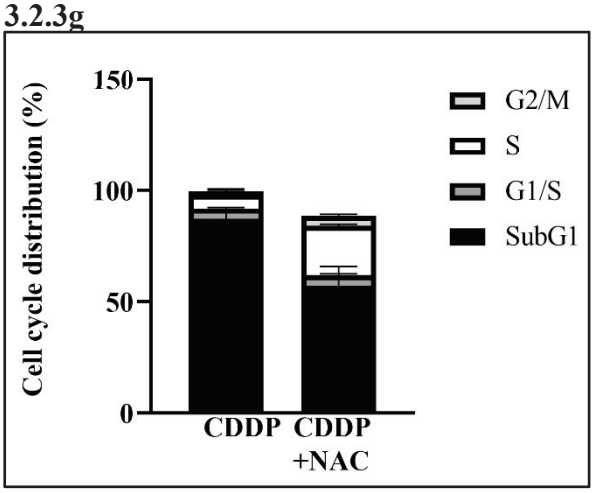
Mean Nuclear Fluorescence Intensity of EZH2 (AU)
200
150
100
50
0
CDDP
CDDP +NAC

3.2.3g



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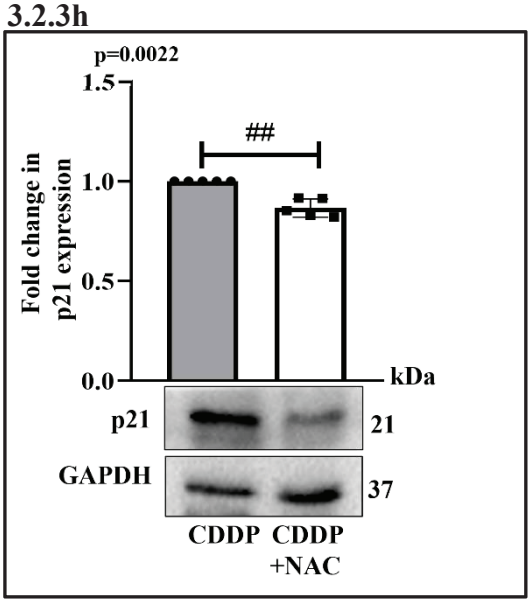
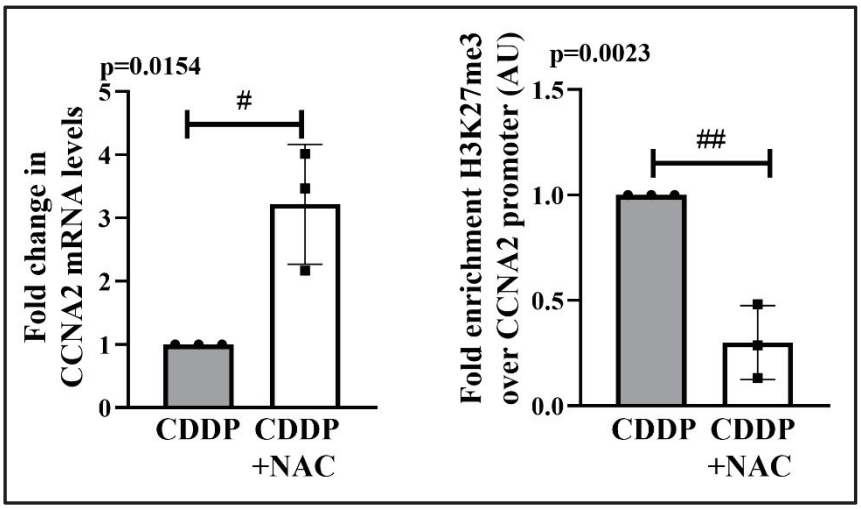


3.2.3i



3.2.3j

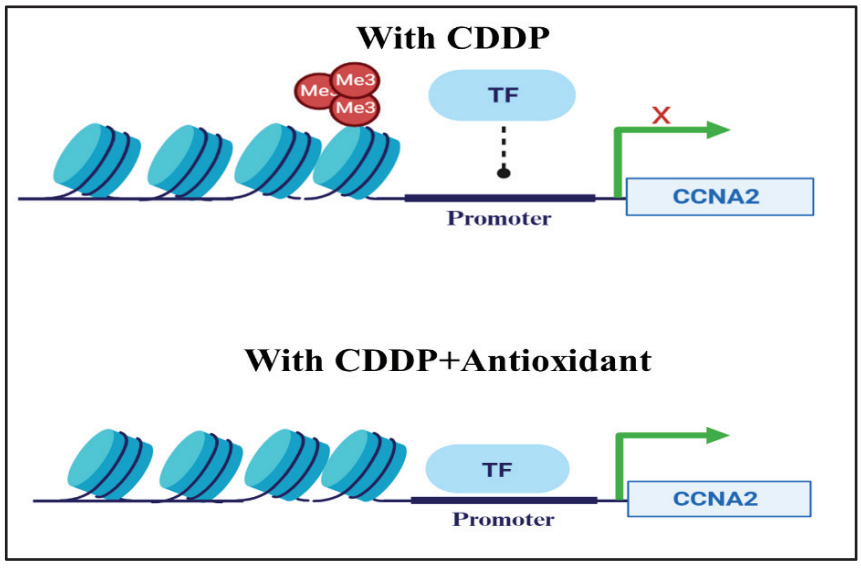


Fig. 3.2.3 ROS regulate repressive epigenomic marks responsible for growth arrest **a)** Reactive Oxygen species levels after incubating cells with CDDP at various time points (24h, 48h, 72h) **b)** Flow cytometric analysis of mitochondrial ROS generation via MitoSox at 24h **c)** Flow cytometric analysis of mitochondrial membrane depolarisation via JC-1 dye at 24h **d)** Immunoblot analysis showing expression of EZH2 and H3K27me3 after removal of oxidative stress (with N-acetyl Cysteine) at 24h; GAPDH and H3 served as the loading control **e, f)** Immunofluorescence staining showing the expression of EZH2 and H3K27me3 at 24h (Scale Bar: 20μm, ImageJ) **g)** Cell cycle analysis through flow cytometry at 24h under CDDP+NAC exposure wrt CDDP treated cells **h)** Immunoblot analysis showing the expression of cell cycle inhibitor, p21 **i)** Change in mRNA expression of cell cycle gene-CCNA2 at 24h and enrichment of H3K27me3 over the cell cycle genes (CCNA2) through ChIP-RTPCR, **j)** Pictorial representation of deposition of H3K27me3, with CDDP and CDDP+NAC. All values are represented as mean±SD; n=3. Unpaired t-test and One way ANOVA, wherever applicable, was applied with (*) $p<0.05$ to estimate the significance when two groups were compared. CTRL represents untreated cells.

3.2.4 YAP is activated and epigenetically regulated post-CDDP-induced ROS generation

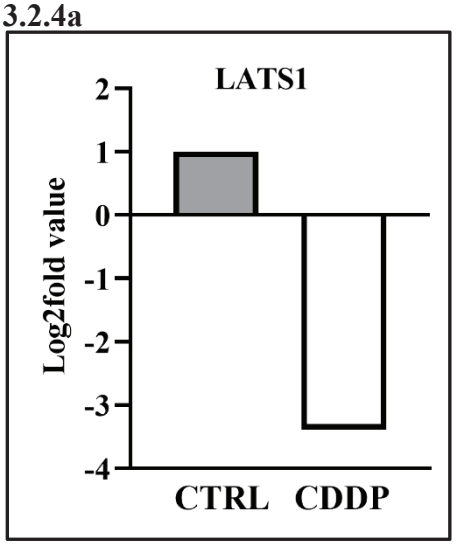
Recent literature and analysis of our RNA sequencing from cells exposed to CDDP provide interesting evidence towards dysregulation of genes involved in maintaining cellular cytoskeletal dynamics (GO:0015630); for example, we observed aberration in expression of genes involved in the Hippo signalling pathway. The latter has a well-established role in contact inhibition, cellular mechanosensing and modulation of the cytoskeleton; and we observed that LATS1- a key protein of the Hippo pathway and a tumour suppressor involved in attenuation of activity of the potent transcription factor – Yes Associated Protein (YAP) - was downregulated (Log2Fold= -3.39; p-value=0.022; GEO Accession Number GSE86053) after CDDP exposure (**Fig.3.2.4a)**. Furthermore, a few reports with existing patient

immunohistochemistry analyses reveal that at least 60-80% of OS patients have high YAP expression, and its aberrant expression is associated with poor prognosis[113, 153, 154]. To corroborate our sequencing findings, we analysed the expression of LATS1 at the translational level and found it to be decreasing (**Fig.3.2.4b)**. To establish the role of YAP in cisplatin-induced cytotoxicity, we further looked at the levels of YAP level post-CDDP treatment. Importantly, CDDP led to a transcriptional increase of YAP **(Fig.3.2.4c)** and its downstream effector proteins- such as CYR61 and CTGF (**Fig.3.2.4d, 3.2.4e).** Importantly, in accordance with our earlier results obtained, quenching of ROS lead to a decrease in YAP (**Fig. 3.2.4f, 3.2.4g)** while a simultaneous increase in LATS1 was also observed post NAC treatment (**Fig.3.2.4h)**. We were also interested in finding whether ROS could affect the transcriptional activity of YAP. Hence, we performed a luciferase assay with YAP responsive-luciferase promoter construct; interestingly, consistent with our mRNA and protein expression data, we found an increase in the activity of YAP post-CDDP treatment, while NAC led to a marked decrease in the luciferase activity, as shown in **Fig.3.2.4i.** This was further substantiated by a decreased expression of CTGF and CYR61 at transcriptional and translational levels respectively **(Fig.3.2.4j, 3.2.4k, 3.2.4l).**

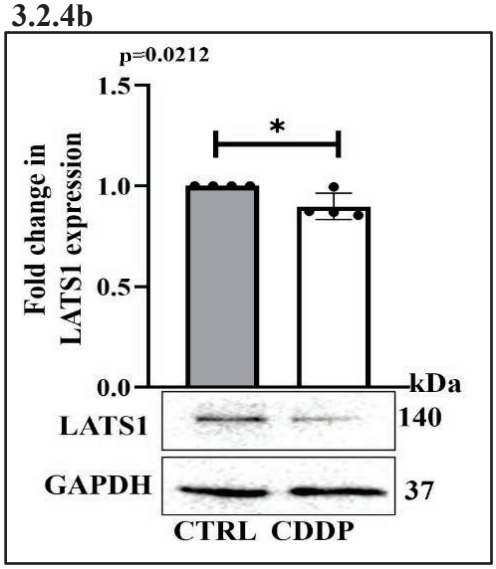
There was a decreased nuclear localization of YAP in presence of NAC **(Fig.3.2.4m)**. The above data clearly indicates that oxidative stress regulates YAP and its activity after cisplatin exposure. Given that CDDP resulted in an increase in repressive mark, the molecular events resulting in a contrasting YAP activation was further interrogated. Importantly, ChIP assay with H3K27me3 pull down showed an enrichment of the repressive mark over LATS1/2 promoter after CDDP treatment **(Fig.3.2.4n),** while NAC treatment showed a reverse effect. This implies that the expression of LATS1/2 was rendered inactive post-CDDP treatment through transcriptionally repressive epigenetic alterations resulting in YAP activation. A completely reverse enrichment was observed over YAP promoter, with low H3K27me3 in presence of

cisplatin, while high repressive mark when NAC added alongside CDDP. Herein, the overall findings clearly suggest that CDDP mediates a ROS-dependent regulation of YAP through gene specific regulation of histone repressive marks. However, the role of increased YAP activity under drug insult remains to be further analyzed.

3.2.4a



3.2.4b



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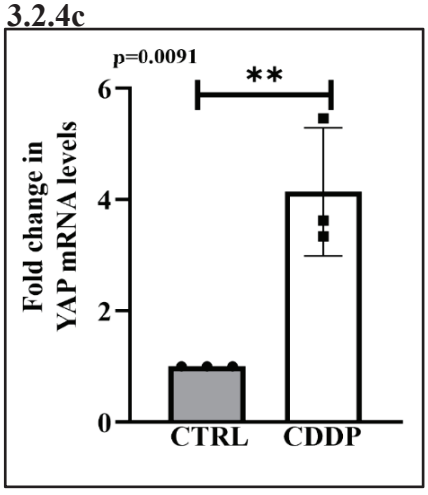
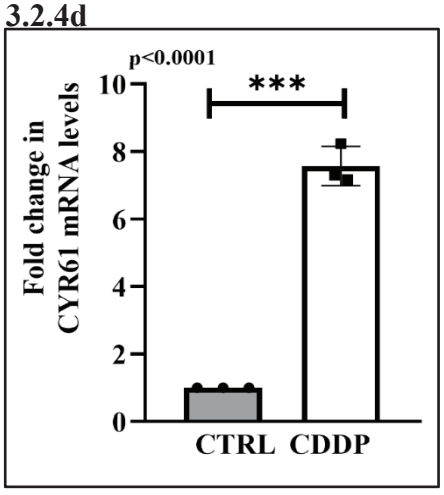
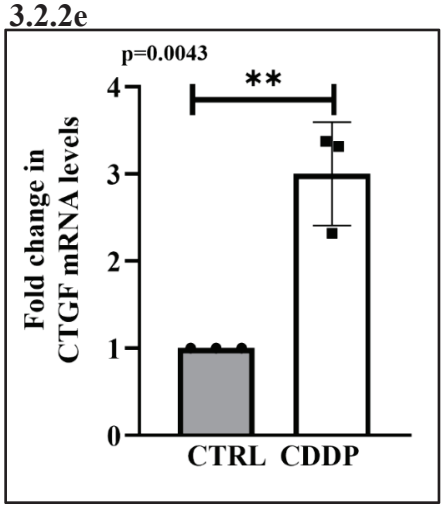


3.2.4d

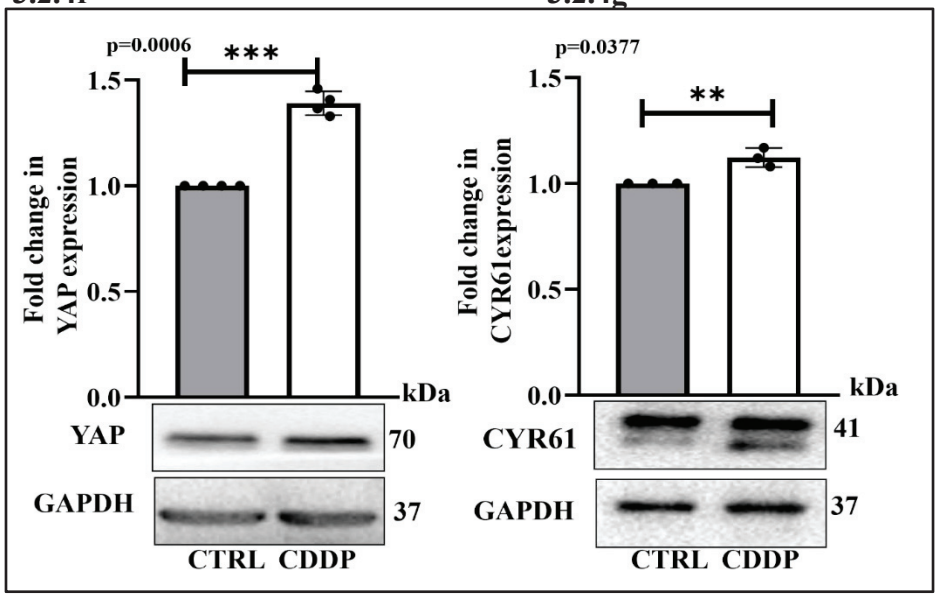


3.2.2e



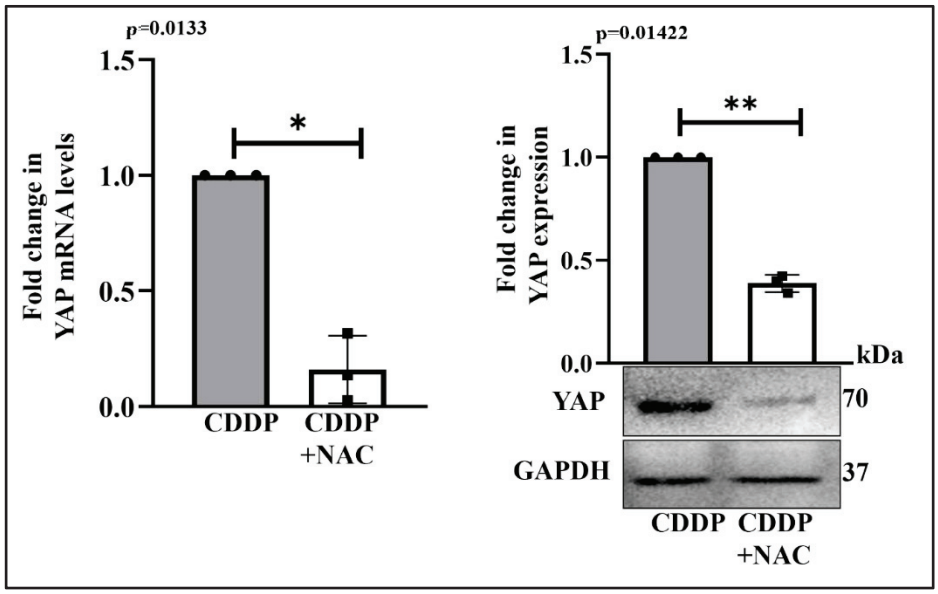
3.2.4f

3.2.4g

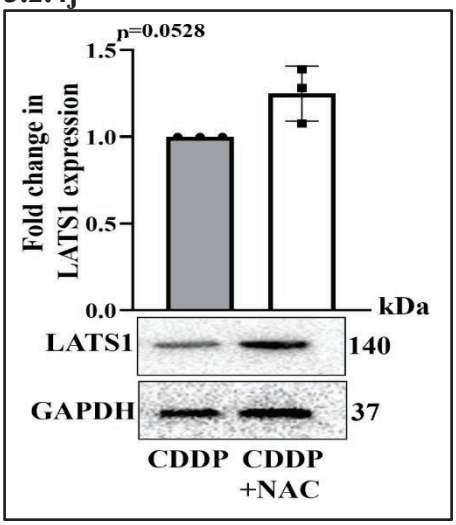


3.2.4h

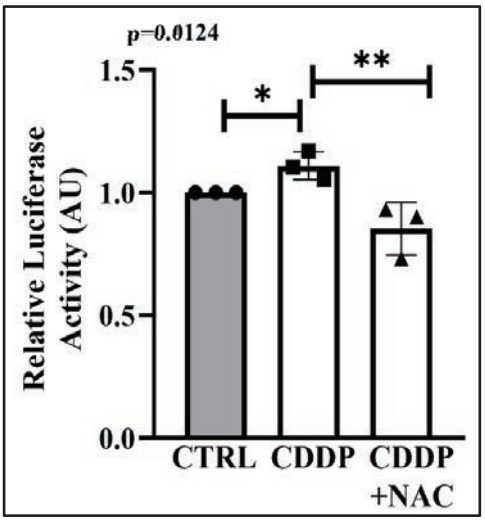
3.2.4i



3.2.4j



3.2.4k



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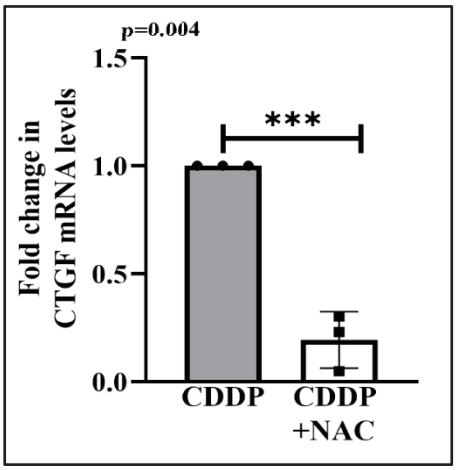

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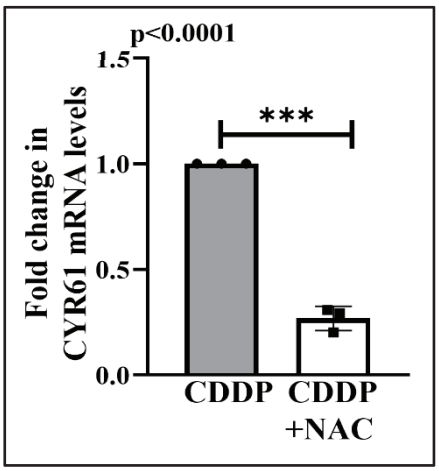

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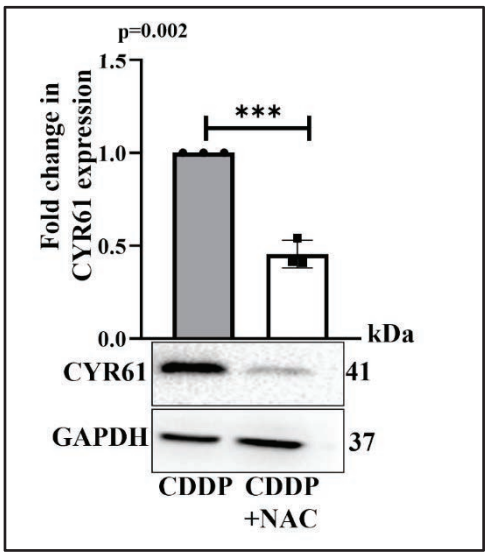

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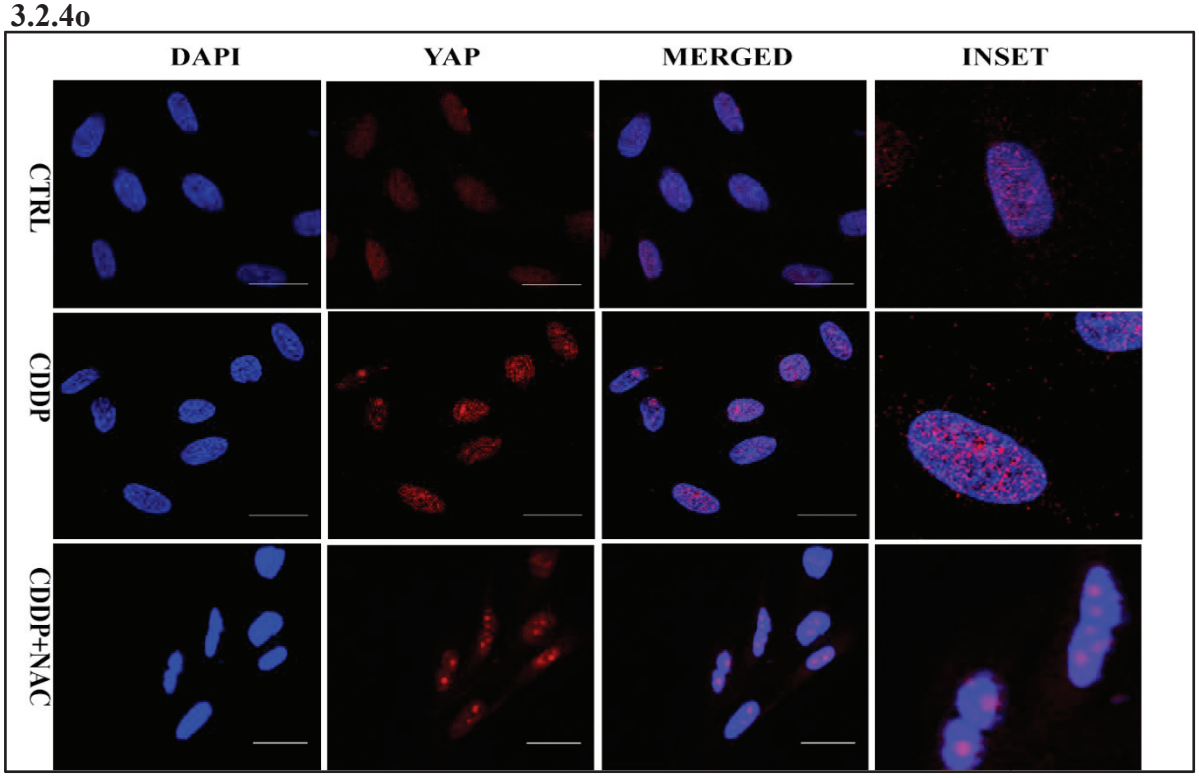
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MERGED
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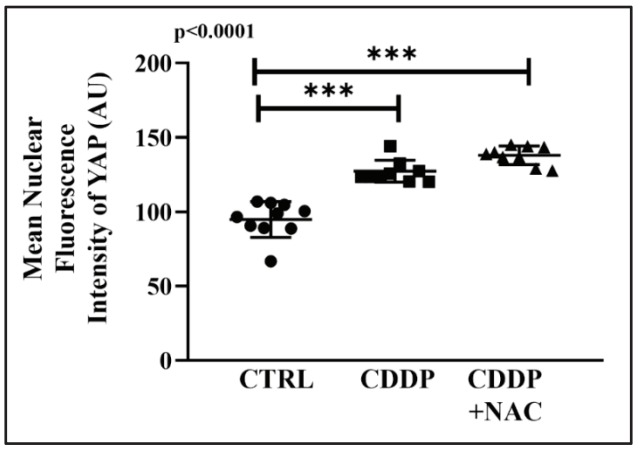
CTRL
CDDP
CDDP+NAC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CTRL
CDDP
CDDP +NAC

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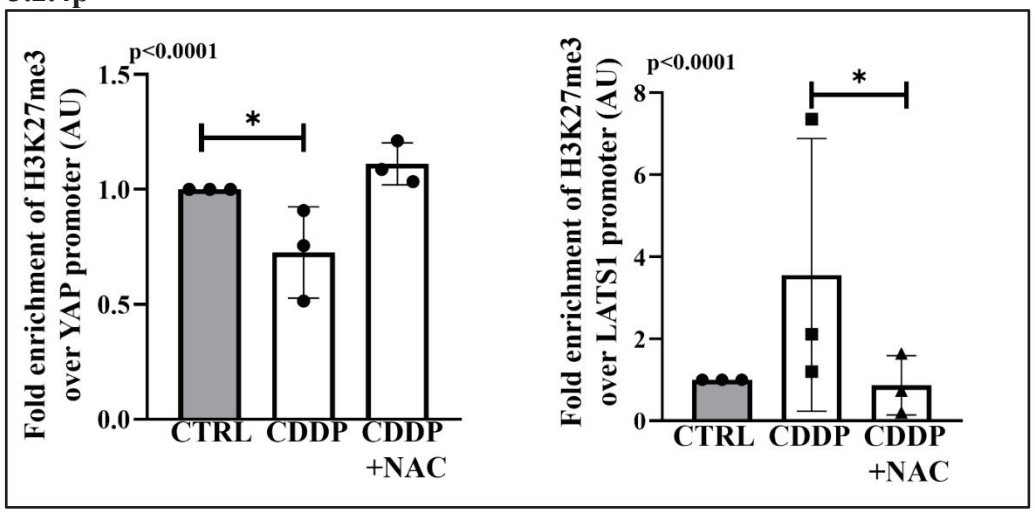
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1.0
0.5
0.0
CTRL
CDDP
CDDP +NAC
p<0.0001
*
Fold enrichment of H3K27me3 over LATS1 promoter (AU)
8
6
4
2
0
CTRL
CDDP
CDDP +NAC

Fig. 3.2.4 YAP is activated and epigenetically regulated post-CDDP-induced ROS generation

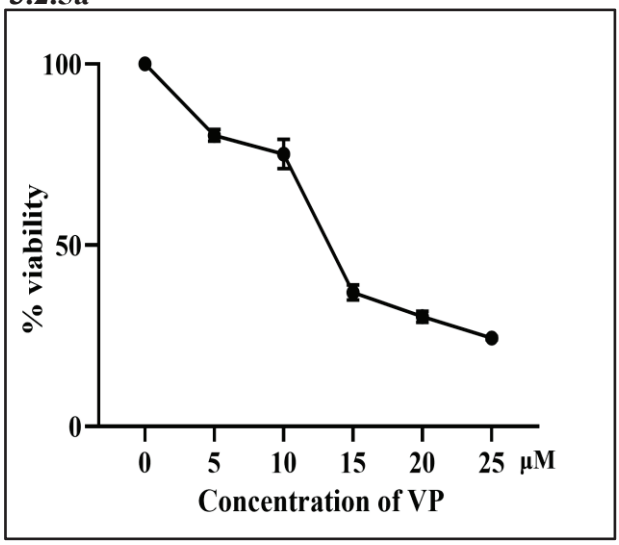
a)Bar graph showing the differential expression of LATS1 post CDDP treatment **b)** Immunoblot analysis showing expression of LATS1 at 24h, GAPDH is the loading control **c, d, e)**Change in mRNA expression of YAP, CYR61, CTGF respectively at 24h when exposed to CDDP **f, g)** Immunoblot analysis showing expression of YAP and CYR61, respectively at 24h, GAPDH is the loading control **h)** Change in mRNA expression of YAP at 24h after ablation of oxidative stress **i)** Immunoblot analysis showing expression of YAP at 24h after NAC treatment, GAPDH is the loading control **j)** Immunoblot analysis showing expression of LATS1 at 24h after NAC treatment, GAPDH is the loading control **k)** Change in Luciferase activity of YAP post exposure to drug treatment at 24h **l, m)** Change in mRNA expression of CTGF and CYR61, respectively at 24h after ablation of oxidative stress **n)** Immunoblot analysis showing expression of CYR61at 24h after NAC treatment, GAPDH is the loading control **o)** Immunofluorescence staining showing the expression of YAP at 24h (Scale Bar: 20μm) **p)** Enrichment of H3K27me3 over the Hippo pathway core genes YAP and LATS1, respectively through ChIP-qPCR. All values are represented as mean±SD; n=3. */#, **/## and ***/### refers to p value significance of ≤0.01, ≤0.001 & ≤0.0001 respectively.

3.2.5 Inhibition of YAP decreases repressive chromatin mark

Existing reports suggest that YAP is involved in epigenomic reprogramming with respect to acetylation via interacting with NuRD complex, however very limited studies have explored the role of YAP in mediating methylation-based changes or its interacting with writers of methylation. Hence to understand the role of YAP in our study, we used the pharmacological inhibitor of YAP- Verteporfin (VP) or performed genetic ablation studies (siRNAs). We firstly did a dose kinetics for 24h for Verteporfin and decided on the dose of 10μM **(Fig.3.2.5a).**

Interestingly, pharmacological inhibition of YAP resulted in a decrease in CDDP-induced global levels of H3K27me3 **(Fig.3.2.5b)**. To disregard any arbitrary drug effect, siRNA-mediated knockdown of YAP was also performed, which also showed similar results with H3K27me3 **(Fig.3.2.5c)**. The led us to hypothesize that YAP is required to induce global and/or specific histone repressive marks post drug insult. Importantly, YAP expression was also positively correlated with EZH2 expression, indicating that YAP regulates EZH2 both at the transcription and protein levels as well (**Fig.3.2.5d, 3.2.5e, 3.2.5f, 3.2.5g**). Furthermore, the nuclear localization of EZH2 evidently decreased after CDDP plus YAP inhibition compared to only CDDP **(Fig.3.2.5h)** corroborating the fact that YAP not only controls the recruitment of repressive methylation marks but also the expression and localization of the methyltransferase-EZH2. Herein, we further performed co-localization studies involving YAP and EZH2 post-CDDP treatment, and importantly, a substantial co-localization of these two proteins was observed post CDDP exposure; VP had a negative effect on the same (**Fig.3.2.5i)**. The above results provide concrete evidences for the role of YAP in regulation of EZH2 expression and its subsequent repressive activity post drug insult in the OS cells. This data suggests that YAP has some association with enhancer of zeste 2homolog, and/or can regulate it. To fully understand whether YAP1 interacts with EZH2 to perform these functions, we performed immunoprecipitation with YAP1 and probed for EZH2, as shown in **Fig.3.2.5j**. Our immunoprecipitation study confirmed that YAP1 and EZH2 proteins physically interact with each other after treatment with CDDP, regulating the fate of OS cells. Importantly, pharmacological inhibition of EZH2 did not significantly alter YAP levels (**Fig.3.2.5l**) suggesting that EZH2 could also be a downstream target of YAP. This set of data consistently provides a key understanding of the involvement of the Hippo/YAP pathway in mediating epigenomic changes to facilitate the survival of OS cells.

3.2.5a



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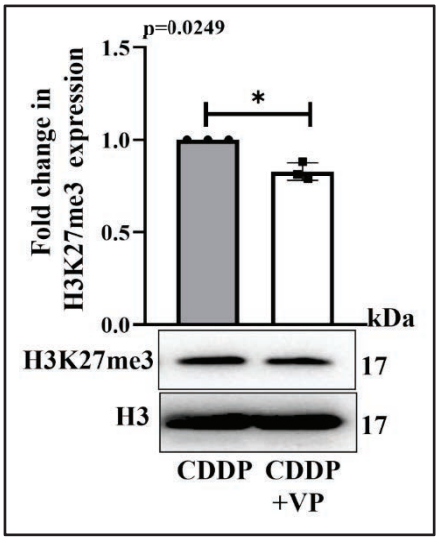

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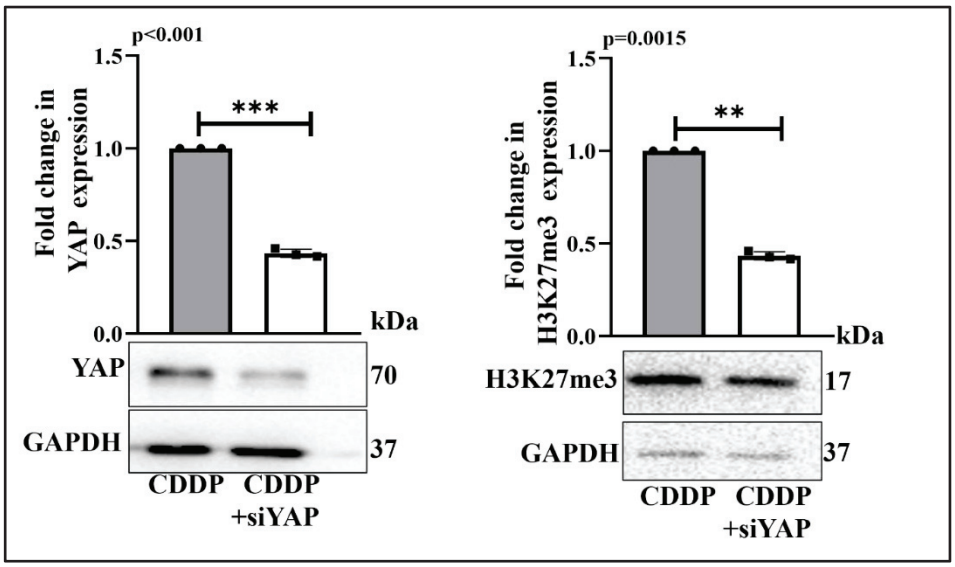

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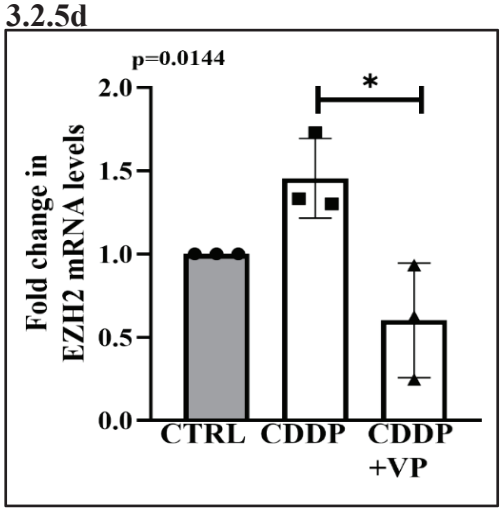

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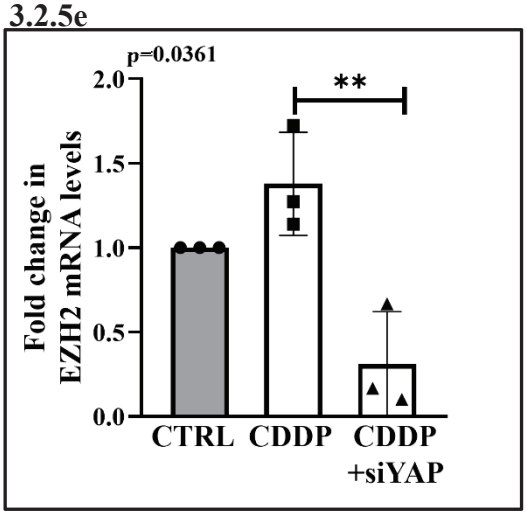

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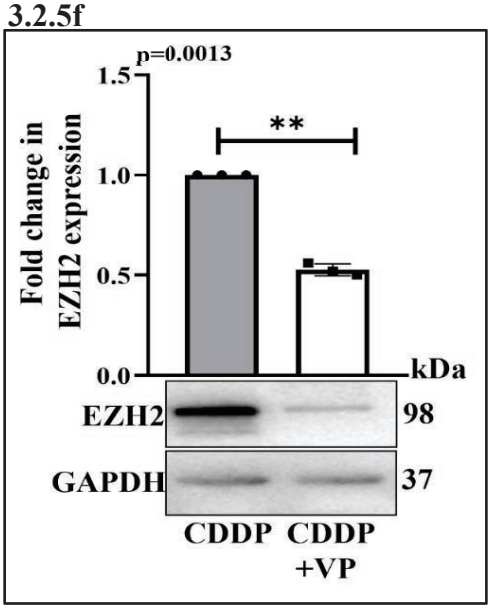

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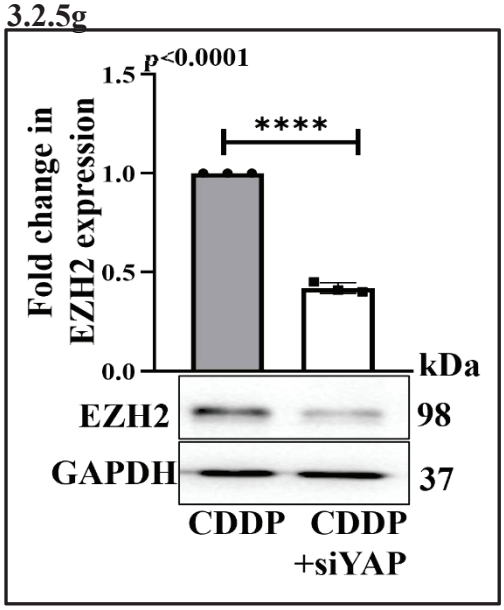

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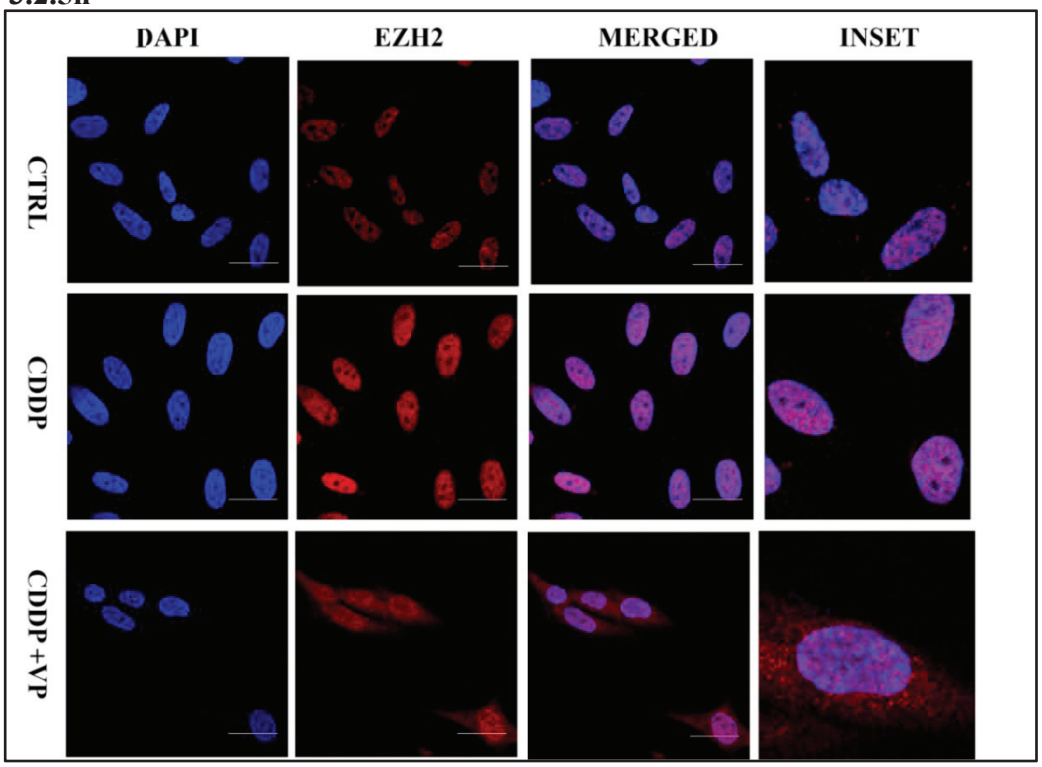
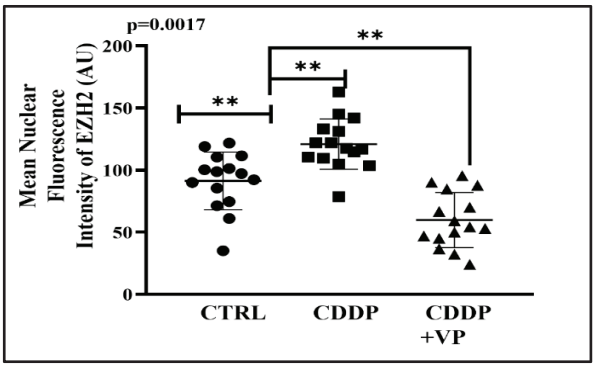
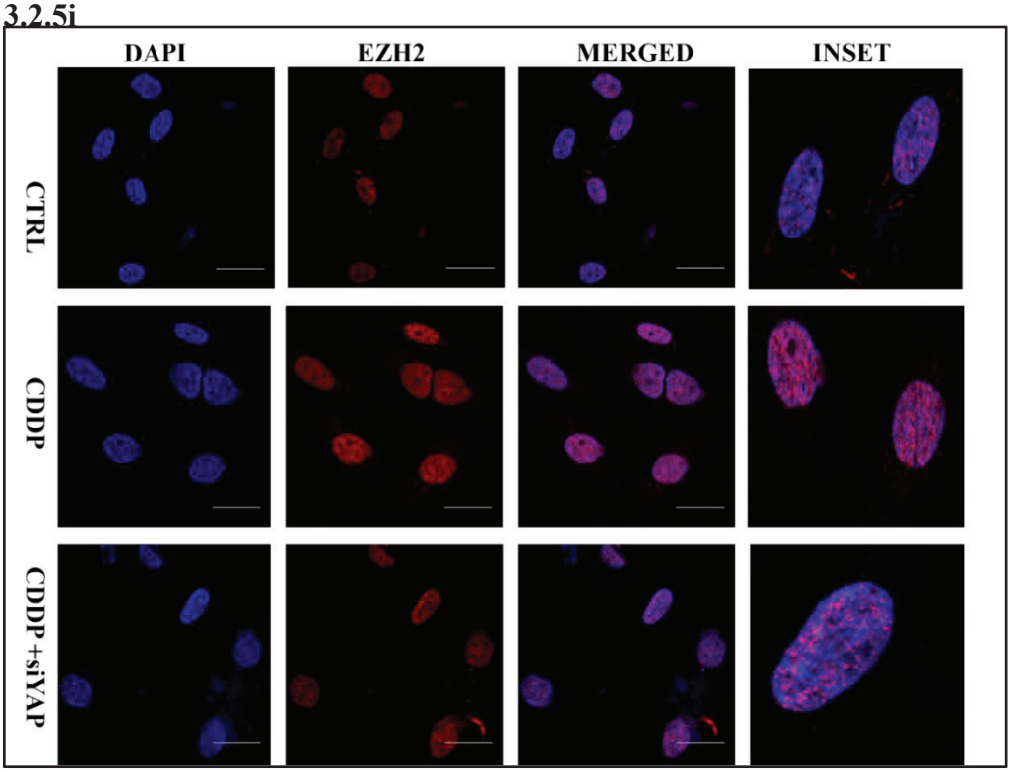




3.2.5i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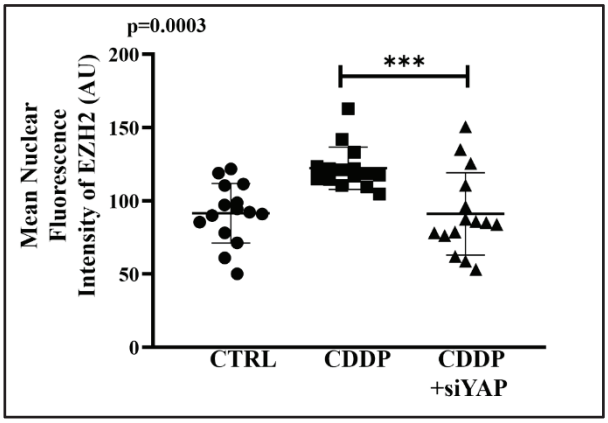
MERGED
INSET
CTRL
CDDP
CDDP+siYAP


p=0.0003
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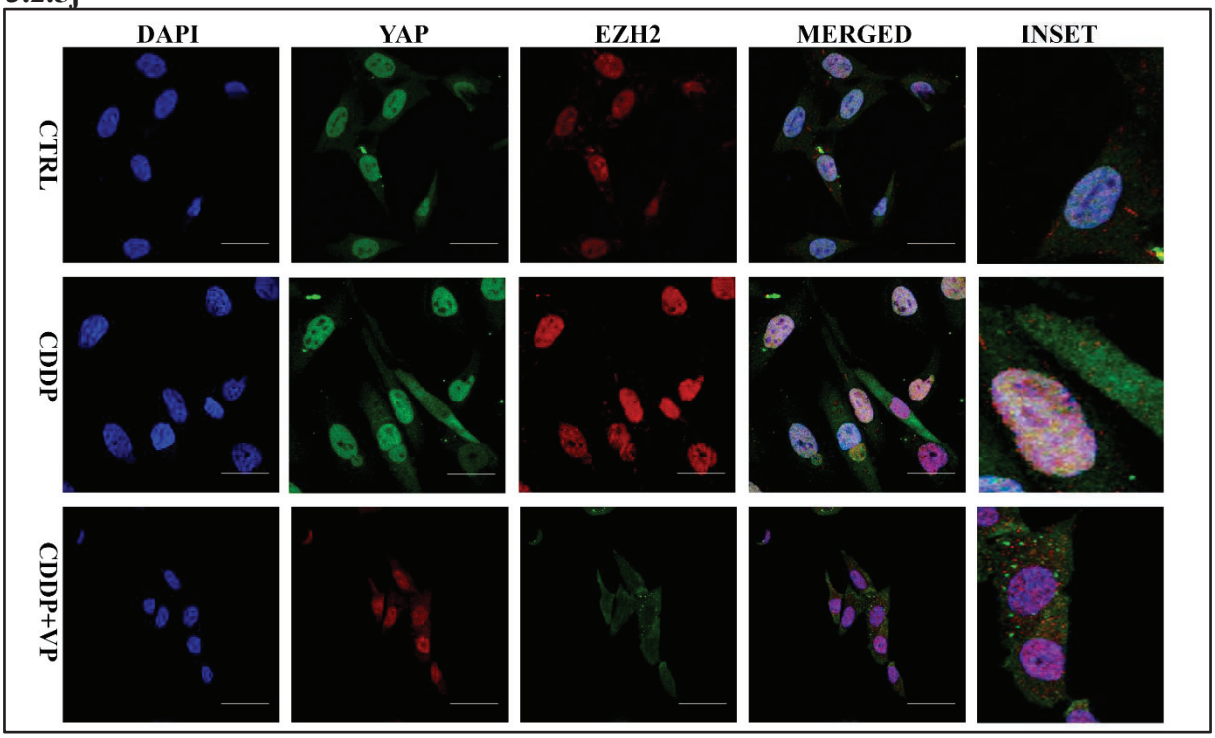
100
50
0
CTRL
CDDP
CDDP +siYAP

3.2.5j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EZH2
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INSET
CTRL
CDDP
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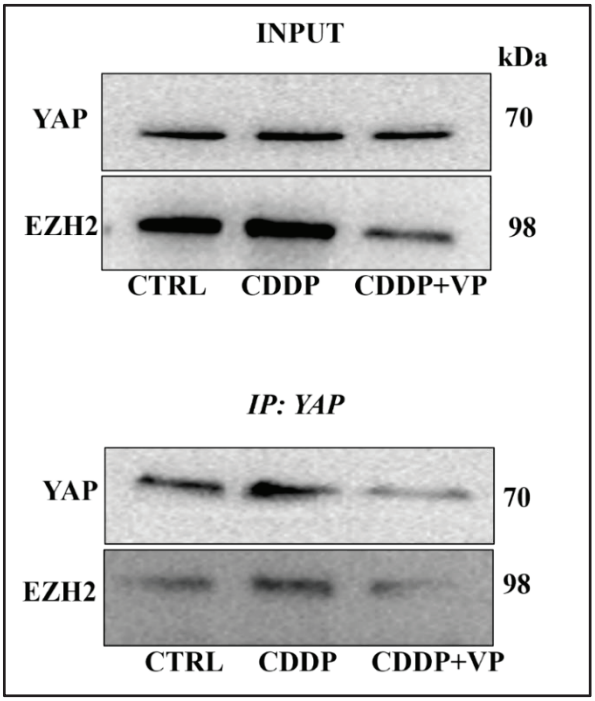
3.2.5k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CDDP+VP

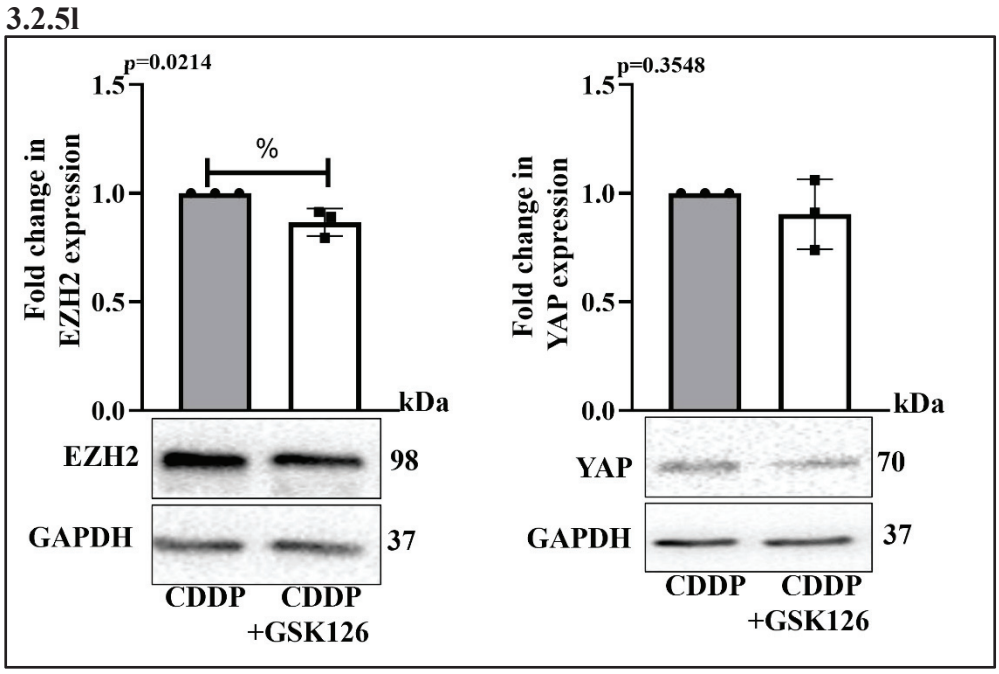
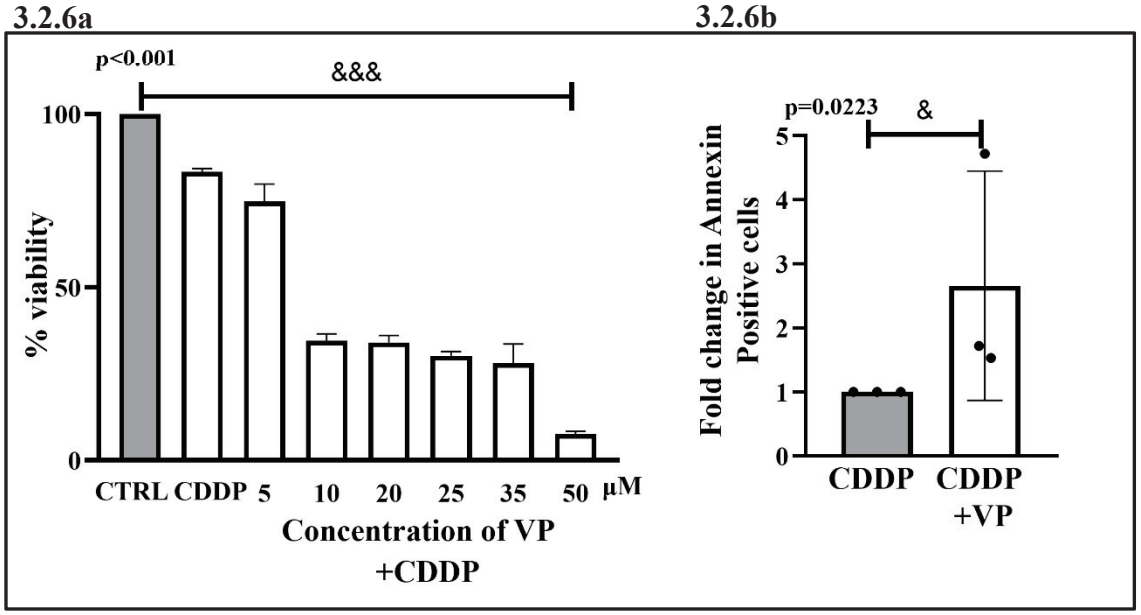


Fig. 3.5 Inhibition of YAP decreases repressive chromatin mark (a) Cellular Viability Assay for Verteporfin for 24h **b)** Change in H3K27me3 expression through immunoblot post-CDDP plus VP (10µM) treatment **c)** Change in YAP and H3k27me3 expression through immunoblot post siRNA treatment **d)** Change in mRNA expression of EZH2 at 24h when YAP is inhibited with VP (10µM) **e)** Change in mRNA expression of EZH2 post-siRNA knockdown of YAP **f)** Immunoblot showing expression of EZH2 after VP treatment at 24h, GAPDH is the loading control **g)** Immunoblot showing expression of EZH2 post siYAP knockdown at 24h, GAPDH is the loading control **h)** Immunofluorescence staining showing the expression of EZH2 after inhibition with VP at 24h (Scale Bar: 20µm) **i)** Immunofluorescence staining showing the expression of EZH2 post siRNA knockdown at 24h (Scale Bar: 20µm) **j)** Immunofluorescence staining showing the expression and co-localisation of YAP and EZH2 at 24h with CDDP and CDDP plus VP (10µM) (Scale Bar: 20µm) **k)** Co-immunoprecipitation immunoblot showing YAP and EZH2 interacting with other **l)** Immunoblot analysis showing expression of EZH2 and YAP at 24h post CDDP plus GSK-126 (25µM) treatment, GAPDH is the loading control.

All values are represented as mean±SD; n=3. &/@, &&/@@ and &&&/@@@ refers to p value significance of ≤0.01, ≤0.001 & ≤0.0001 respectively.

3.2.6 Inhibition of YAP and/or EZH2 as a potent strategy to sensitize OS cells

Since combination drug therapies are now predominantly being used as treatment strategy against multiple cancers, our finding provides scope for modulating the therapeutic strategy against OS for better outcomes. Herein, we show that inhibiting YAP through Verteporfin (10μM) causes the cells to become more sensitive to low doses of the drug CDDP, as observed through MTT assay (**Fig.3.2.6a**). We further performed AnnexinV-PI staining to confirm our observation, and a marked increase in the number of Annexin positive cells was observed after combinatorial treatment of VP with CDDP **(Fig.3.2.6b)**. Importantly, inhibition of YAP with the FDA approved drug – VP was found to have prominently more cytotoxic effect than EZH2 inhibition with GSK126 (25μM) on the OS cells studied **(Fig.3.2.6c, 3.2.6d)**. The above findings provide a scope to offset the deleterious effects of high dose CDDP chemotherapy on the patients and propose that a low dose of CDDP in combination with the FDA approved YAP inhibitor- VP can be an alternative efficacious therapy.



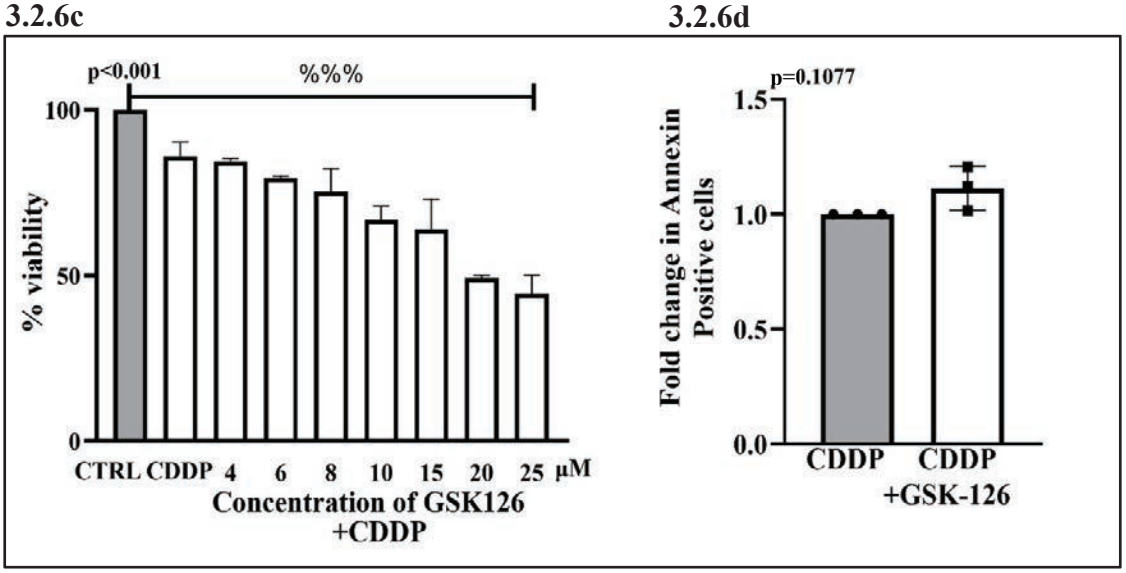


Fig. 3.6 Inhibition of YAP and/or EZH2 as a potent strategy to sensitize OS cells **a)** Cell Viability (MTT) assay at different concentrations of VP and CDDP (3µM) for 24h **b)** Bar graph indicating the number of Annexin positive cells in CDDP plus VP(10µM), CDDP is used as a control **c)** Cell Viability (MTT) assay at different concentrations of GSK-126 and CDDP (3µM) for 24h **d)** Bar graph depicting the number of Annexin positive cells in CDDP plus GSK-126 (25µM), CDDP is used as a control. All values are represented as mean±SD; n=3. %/&/$, %%/&&/$$ and %%%/&&&/$$$ refers to p value significance of ≤0.01, ≤0.001 & ≤0.0001 respectively.

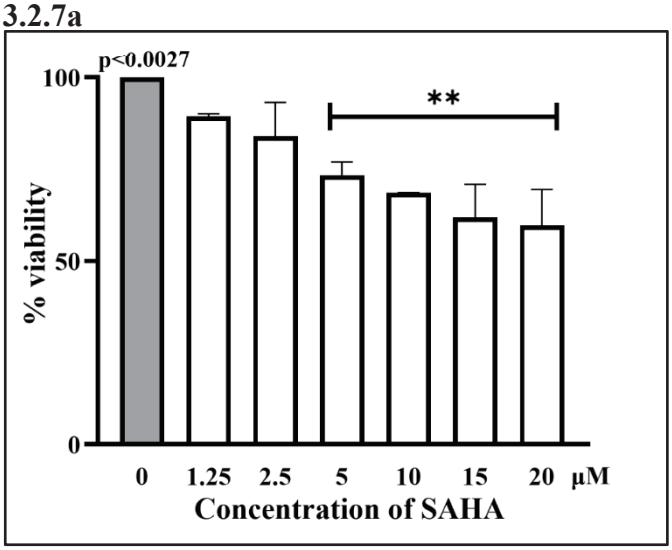
3.2.7 HDACi suppresses YAP activity to enhance the cellular sensitivity of CDDP

Herein, based on our current observations, we further envisaged that since ROS-induced YAP aids in the recruitment of repressive epigenomic marks post CDDP exposure, another alternate strategy could be inhibition of the histone deacetylases which remove acetyl groups from the promoter of genes to induce transcriptomic repression. We did a cell viability assay for SAHA and chose 15µM as our sub-lethal dose of HDAC inhibitor (**Fig.3.2.7a**). Interestingly, as shown in **Fig.3.2.7b**, we see a combined treatment of CDDP and SAHA reduces cell viability to ~40% vis-à-vis individual drugs. We also performed synergism analysis using CompuSyn software **(Fig.3.2.7c)** and saw a combinatorial index <1 for all the tried combinations, suggesting the

synergistic effect of SAHA. All these findings were substantiated by Annexin PI analysis (**Fig.3.2.7d)** and crystal violet assay (**Fig.3.2.7e).** Interestingly, we also observed a decreased expression of H3K27me3 & H3K9me3 while a simultaneous increase in H3K27ac and H3K9ac was also observed post-combinatorial treatment of CDDP and SAHA (a pan-HDAC inhibitor; 15μM) (**Fig.3.2.7f, 3.2.7g)**. In corroboration to the above, inhibition of HDACs could also be a potent treatment regimen to sensitize OS cells to the drug CDDP. Since HDACi has shown a promising target, we were interested whether HDAC inhibition can have any effect on either YAP or EZH2, the two key molecules of our study. As shown in **Fig.3.2.7h**, we see that with the inhibition of HDACs, there is a decrease in the transcriptional activity of YAP and its downstream targets, CYR61 and CTGF. Similarly, at translational levels, we see YAP's expression goes down in the combinatorial treatment **(Fig.3.2.7i).** Interestingly, the expression of EZH2 also went down in the combinatorial treatment **(Fig.3.2.7j).** This is intriguing to note as HDAC inhibitors aim towards a more open chromatin, affecting the expression of EZH2 could be a synergistic effect that they may be exerting. Immunofluorescence staining (**Fig.3.2.7k)** also proves our hypothesis that HDAC inhibition causes decreased YAP expression. Study by Han Han. Et al. [155] also summarises the effect of HDAC inhibition, stating that Hippo deficiency makes tumours vulnerable to HDAC inhibitors. And that HDAC inhibitors affect YAP and not TAZ expression in a more dose-dependent manner. Consistent with this set of published data, we additionally show that HDAC inhibitor affects the activity of YAP as well, through Luciferase assay **(Fig.3.2.7l).** Various solid malignancies have shown activation of YAP/TAZ, and genetic evidence have confidently elucidated the role of the Hippo/YAP pathway in mediating drug resistance/tolerance leading to relapse. These reports postulate that YAP may be a master transcriptional regulator, acting as a switch to help cells evade drug abuse. In different cancer models, YAP overexpression has been implicated in their metastatic potential[156]. Consistent with existing studies, our study also shows that cisplatin

exposure increases the activity of YAP. Herein, we wanted to explore the possibility of epigenetic regulation of YAP and also the latter meddling with the epigenome to modulate cellular sensitivity. Interestingly we show that YAP activity in CDDP exposed cells is regulated through H3K27me3 mediated silencing of its negative regulator LATS. Furthermore, YAP has also been reported to work with the NuRD complex to deacetylate the promoter of specific genes for PRC2 to be recruited for gene repression. Our study shows that YAP localizes majorly in nucleus which is consistent with patient and murine models. We also show shutting YAP off prevents EZH2 activity as well. We further show YAP and EZH2 to co-localize with each other while the knockdown of YAP reduces EZH2 nuclear localization. Additionally, downregulation of YAP or even ROS quenching, ultimately affects EZH2 expression patterns. Hence, we postulate that YAP is upstream of EZH2 and drives tumor adaptation to drug stress via EZH2-mediated repression of genes. Overall, our study presents an alternative treatment regimen to treat a rather uncommon but aggressive cancer- osteosarcoma- by employing the use of combinatorial therapy (CDDP plus YAP inhibitor), allowing the circumvention of the harmful effects of high doses of traditional chemotherapeutic drugs.

3.2.7a



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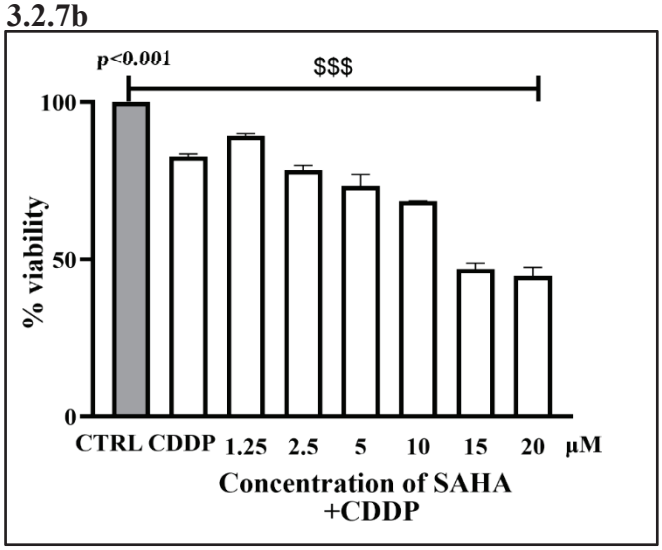

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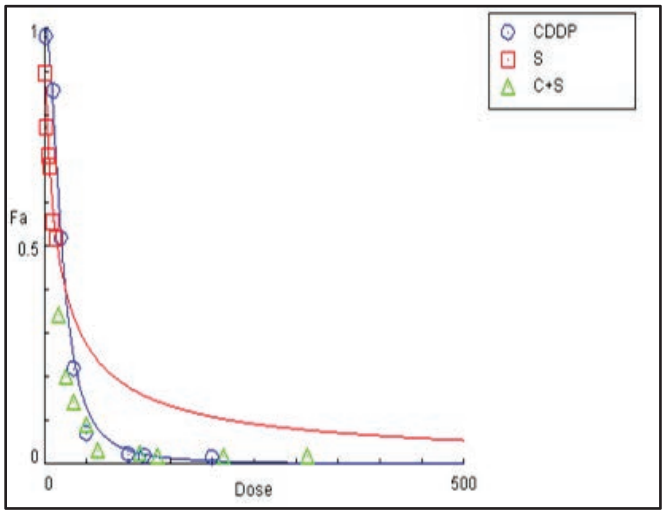

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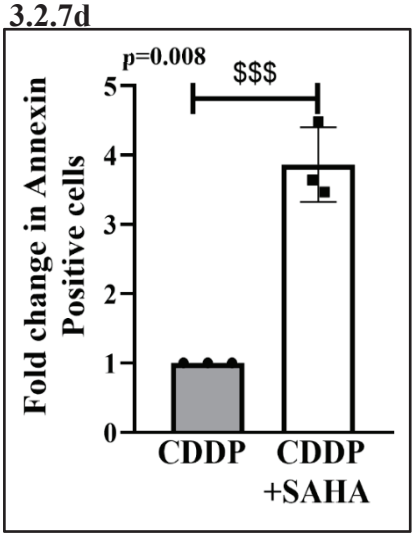

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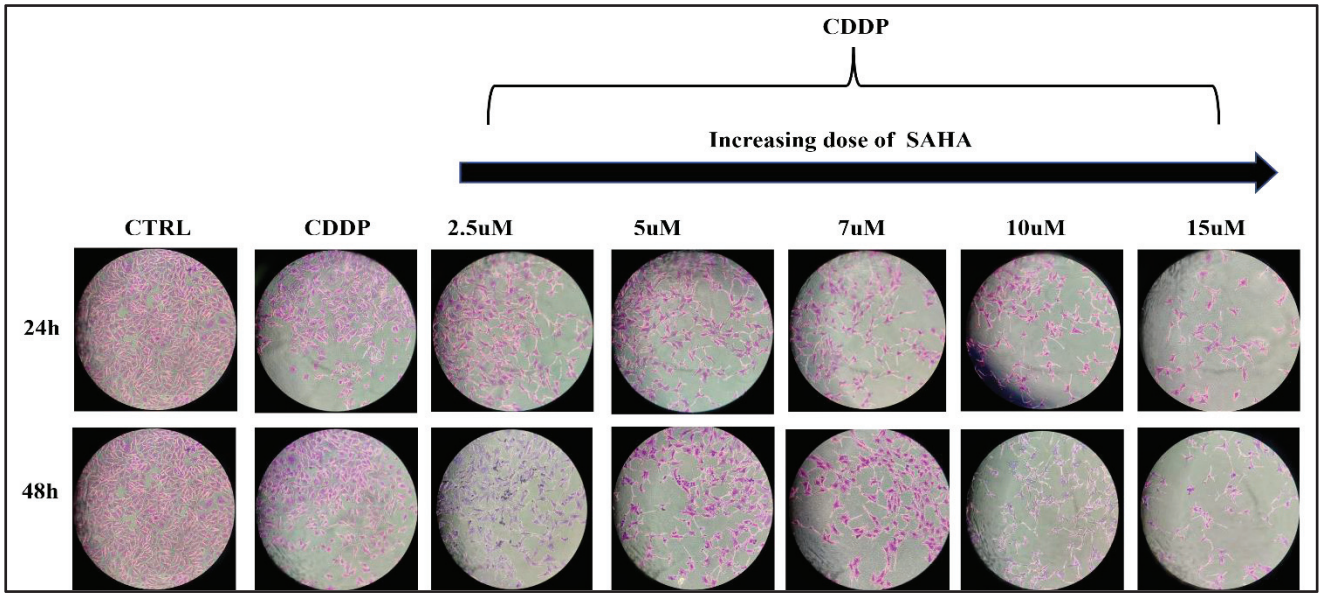

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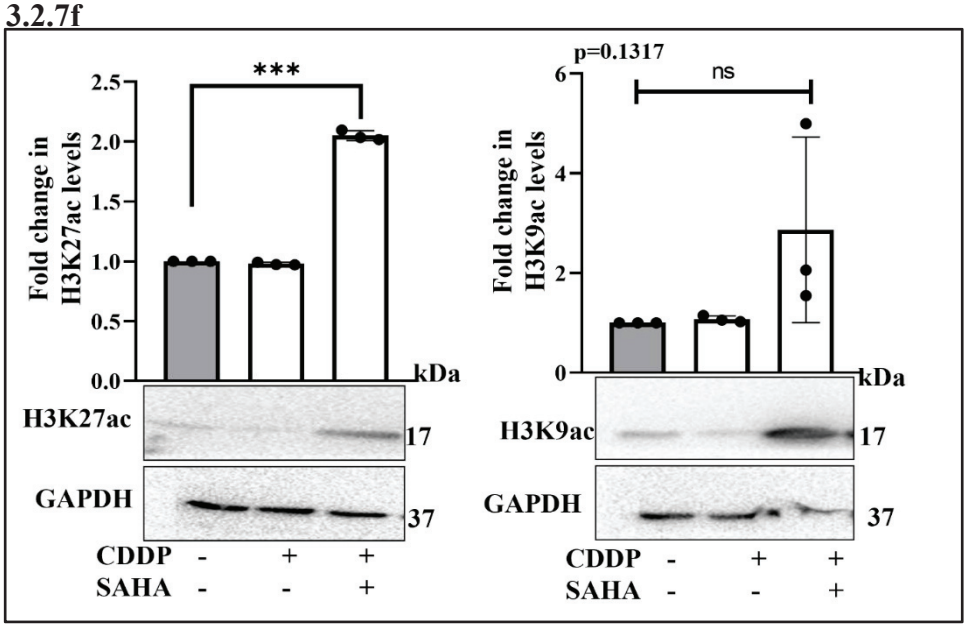
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0.5
0.0
kDa
H3K27ac
17
GAPDH
37
CDDP - + +
SAHA - - +
p=0.1317
ns
Fold change in H3K9ac levels
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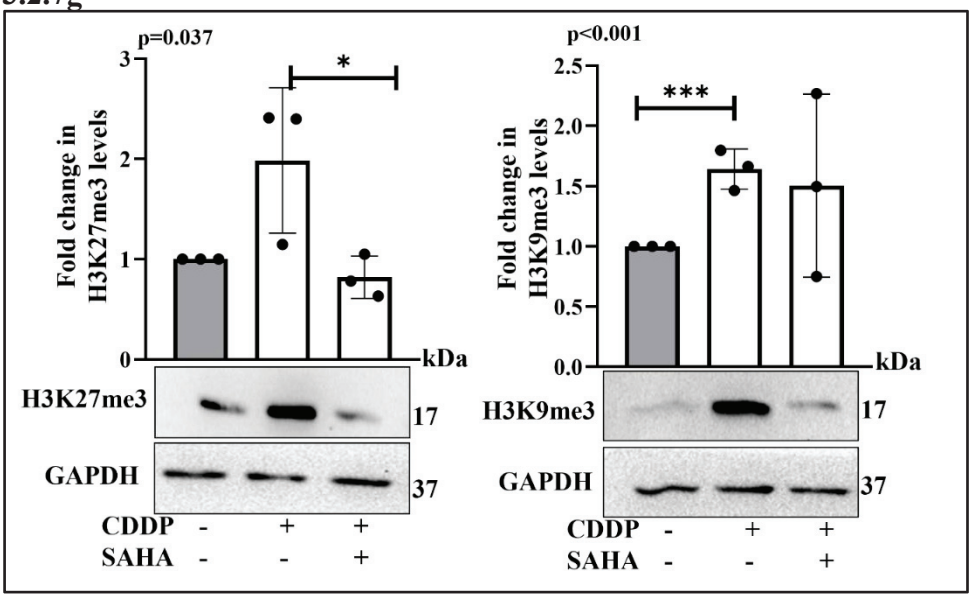
4
2
0
kDa
H3K9ac
17
GAPDH
37
CDDP - + +
SAHA - - +

3.2.7g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kDa
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17
GAPDH
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CDDP - + +
SAHA - - +
p<0.001
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2.0
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GAPDH
37
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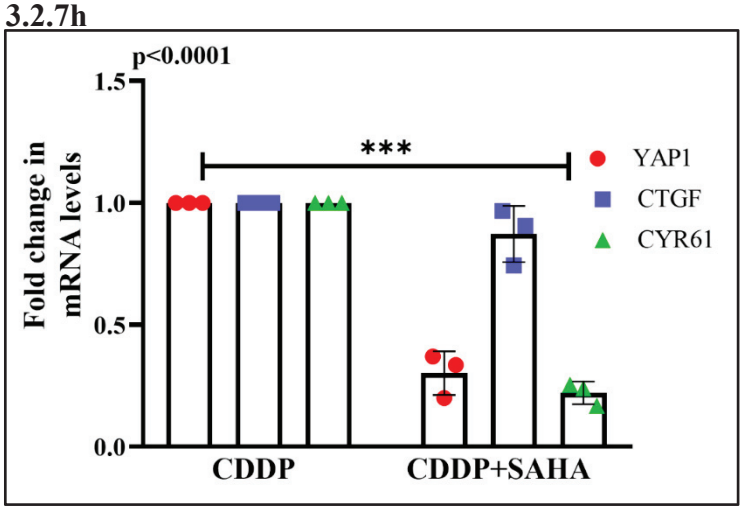
SAHA - - +

3.2.7h



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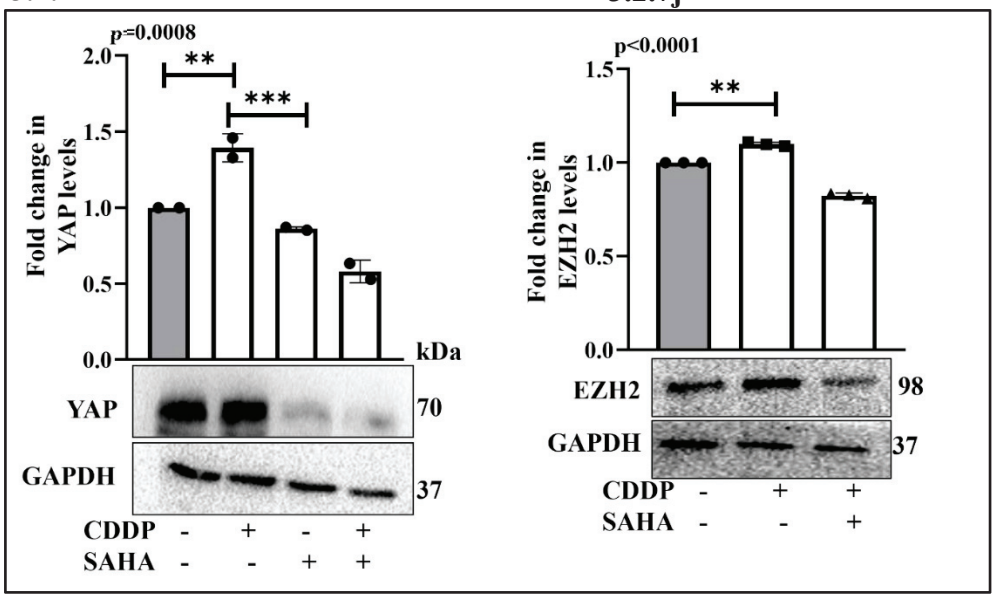
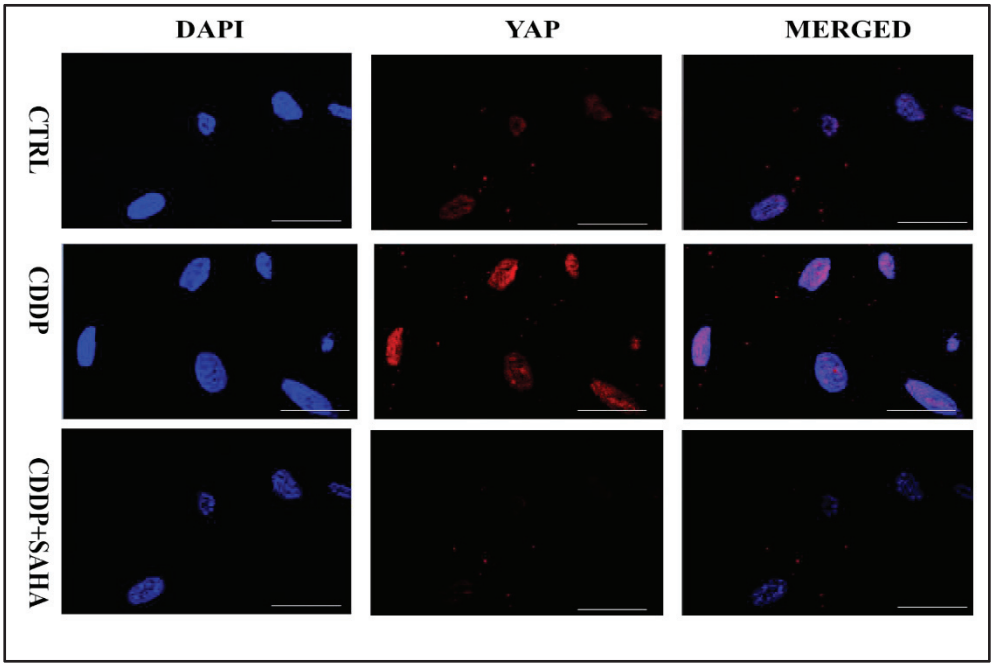
3.2.7j



3.2.7k



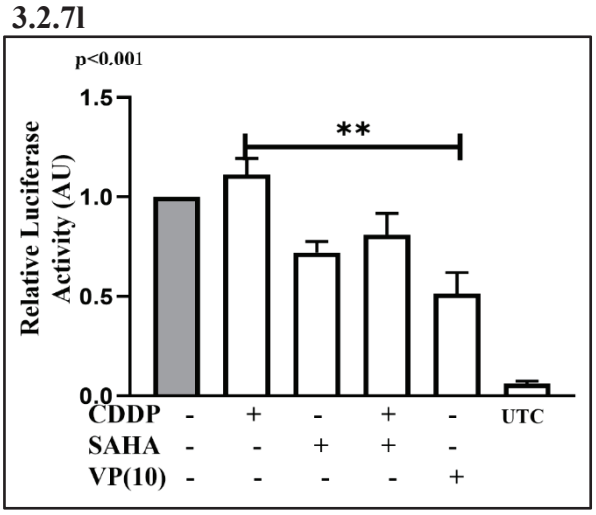


Fig.3.7 HDACi suppresses YAP activity to enhance the cellular sensitivity of CDDP **a)** Cell Viability (MTT) assay at different concentrations of SAHA for 24h **b)** Cell Viability (MTT) Assay with different doses of SAHA and CDDP (3 μM) **c)** Curve showing the synergy of SAHA and CDDP, calculated using CompuSyn software **d)** Bar graph indicating the number of Annexin positive cells in CDDP plus SAHA (15μM), CDDP is used as a control **e)** Crystal Violet imaging qualitatively showing the synergism **f)** Change in H3K27me3 and H3K9me3 expression through immunoblot post CDDP plus SAHA (15μM) **g)** Change in H3K27ac and H3K9ac expression through immunoblot post CDDP plus SAHA (15μM) treatment **h)** Change in mRNA expression of YAP, CYR61, CTGF post CDDP plus SAHA treatment **i, j)**Change in expression of YAP and EZH2, respectively through immunoblot post CDDP plus SAHA (15μM) treatment **k)** Immunofluorescence staining showing the expression of YAP at 24h (scale bar: 20μm, ImageJ) **l)** Luciferase activity assay for YAP at 24hAll values are represented as mean±SD; n=3. %/&/$, %%/&&/$$ and %%%/&&&/$$$ refers to p value significance of ≤0.01, ≤0.001 & ≤0.0001 respectively.

3.3 Discussion:

Cisplatin has traditionally been used as a first line of chemotherapeutics for decades now. Yet, a major hindrance to cisplatin therapy has been the intrinsic or acquired resistance in patients. Even though platinum drug re-treatments have garnered much attention, there remains a loophole in precisely understanding which combinations can work, and/or whether they are ubiquitous across cancers. A few meta-analyses studies have also favored the use of a lower dose regimen for it has potent anti-tumor properties[149, 157], and that cumulative lower dose treatments might be used as adjuvant therapy or as a combination with other modulators. Currently, no adequate therapeutic regimen for OS patients exists and usually a combination or single agent chemotherapies are used, hence in-depth analysis of molecular signature(s)/landscape of OS is crucial to develop an appropriate strategy to counteract its progression.

Global epigenetic changes in cancer cells are one window of opportunity to create drugs that could potentially be less harmful or could prove to be better neo-adjuvants than the current ones. Complex interplay of different epigenetic proteins: writers, erasers and readers set up the tumour milieu albeit differentially across cancer subtypes. Despite them being used for hematological malignancies, their efficacy in solid tumors still is at lapse. The additional effect of non histone proteins, lack of specificity along with their pertinent dynamicity is a considerable drawback. While DNMTi and HDACi have been prolonged used now, the effect of methylation over histones becomes complicated owing to multiple sites and degrees of methylation. Since it is not exactly methylation but rather the readers altering the chromatin by recruiting other factors, it becomes imperative to understand their crosstalk with the factors that they recruit or get recruited by. In this regard, a targeted therapy approach for EZH2 inhibitors has been utilized in lymphomas. Tazemetostat- an EZH2 inhibitor- has been approved for the treatment of metastatic epithelioid sarcoma in pediatric patients in January

2020[158]. Henceforth, epidrugs can be used to prime the cells for the main chemotherapeutic agent leading to synergistic effects, but with limited toxicity. Interestingly, the sequence in which the drugs are added also affects the efficacy of the treatment; for example, in *in-vitro* and *in-vivo* models of NSCLC patients, when pan-HDACi Givinostat was given first before Pemetrexed, it showed better chemosensitivity unlike when Pemetrexed was given first[159]. This does suggest that platinum based or other drugs do change the epigenomic landscape of a tumour cell and priming the cells with epigenetic inhibitors, may lead to activation of tumour suppressor genes that were rendered inactive and can now act on to sensitize these cells to drug assault. Many such new small molecules are now being designed and tested for their efficacy to be a helpful agent in increasing the survival of patients.

However, chromatin modifying enzymes can themselves be modulated in a given tumour microenvironment either by the cytokines or by the generation of superoxides. When it comes to OS, oxidative balance is crucial to maintaining bone remodeling and can affect the pathophysiology of such connective tissues[152]. As is known, micromolar levels of ROS can regulate key processes such as proliferation, excessive levels of ROS can promote tumour progression and development of malignancies. However, newer reports suggest that some tumour cells have the capability to reset their antioxidant defense system in case of higher oxidative stress; the ‘reset’ promotes drug resistance[160], especially in advanced stages of tumour. Hence, for tumour cells that have adapted to higher levels of oxidative stress, single agent therapies, especially platinum-based drugs might not be effective. In lieu of this, the role of oxidative stress in mediating epigenomic changes is now being extensively studied. Dysregulation of oxidative balance can dysregulate both at the level of histones (HDAC1, HMT1, and HAT1) and of DNA methylation enzymes (DNMT1, DNMT3a, and MBD4) [161]. Several studies link GSH (antioxidant enzyme) with epigenomic changes; alteration in

synthesis or depletion of GSH has been linked with global DNA hypomethylation[162]. Many other reports posit that the hydroxyl radicals cause interference in DNMTs-DNA binding capacity thereby leading to global hypomethylation. Recent reports on cardiovascular and other diseases have implicated a role of oxidative stress in modulating the heterochromatin[163]. Oxidative stress works like a catalyst both at the levels of histone substrates and their respective enzymes. A few reports suggest that JmjC domain containing histone demethylases use Fe(II) and α-ketoglutarate (αKG) as cofactors in an oxidative demethylation reaction via hydroxymethyl lysine. Now under oxidative stress, these cofactors are rendered non-functional thereby increasing methylation levels. Removal of these oxidative stressors allows decreasing the expression of H3K27me3. And precisely our study shows that even a sub toxic dose of a platinum drug can induce repressive histone marks that can be reversed if the oxidative stressor is removed. Our observations emphasizes the role of a system like intracellular ROS in targeting the epigenome regulating gene expression. As a result, epi-drugs have a better appeal at circumventing the old age problem of resistance. However, the genes regulated by the epigenetic alterations, especially H3K27me3 after CDDP exposure are till date poorly explored.

Herein, different solid malignancies have shown activation of YAP/TAZ, and genetic evidence have confidently elucidated the role of the Hippo/YAP pathway in mediating drug resistance/tolerance leading to relapse[164]. These reports postulate that YAP may be a master transcriptional regulator, acting as a switch to help cells evade drug abuse. In different cancer models, YAP overexpression has been implicated in their metastatic potential. Consistent with existing studies, our study also shows that cisplatin exposure increases the activity of YAP. Herein, we wanted to explore the possibility of epigenetic regulation of YAP and also the latter meddling with the epigenome to modulate cellular sensitivity. Interestingly we show that YAP activity in CDDP exposed cells is regulated through H3K27me3 mediated silencing of its

negative regulator LATS. Furthermore, YAP has also been reported to work with the NuRD complex to deacetylate the promoter of specific genes for PRC2 to be recruited for gene repression. Our study shows that YAP localizes majorly in nucleus which is consistent with patient and murine models. We also show shutting YAP off prevents EZH2 activity as well. We further show YAP and EZH2 to co-localize with each other while the knockdown of YAP reduces EZH2 nuclear localization. Additionally, downregulation of YAP or even ROS quenching, ultimately affects EZH2 expression patterns. Hence, we postulate that YAP is upstream of EZH2 and drives tumor adaptation to drug stress via EZH2-mediated repression of genes. Overall, our study presents an alternative treatment regimen to treat a rather uncommon but aggressive cancer- osteosarcoma- by employing the use of combinatorial therapy (CDDP plus YAP inhibitor), allowing the circumvention of the harmful effects of high doses of traditional chemotherapeutic drugs.

Chapter 4:
Conclusions, Limitations & Future perspectives

4.1 Conclusions:

Since OS is a rare cancer, molecular alterations of the OS tumour landscape are not well established. Although the majority of OS is sporadic, most mutations that have been studied are either non-targetable or have limited feasibility with respect to clinical trials. Hence, the need of the hour is to invest in researching for potential targeted therapies. The last decade has seen a massive boom in the use of small molecule inhibitors or epidrugs as neo-adjuvants to enhance the effectiveness of primary chemotherapeutic drugs alongside offsetting the various side effects of using high dosages of these anti-cancer drugs. Exploiting the differential vulnerabilities of cancer cells, restrictive combinations (RC) of drugs are now an emerging approach. However, the different phase clinical trials have been empirical in nature, hence while this approach seems feasible in theory, for practical purposes, we need to exhaustively understand and categorise the molecular drivers/events that allow the cells to become resistant to drugs. Herein, our study also emphasizes the dynamic alteration of epigenome under drug stress, identifies the putative histone modifiers thus categorising them as a hub of targetable molecules. As a regulator of chromatin, we identify the disruption of cellular redox balance. Herein, ROS has been mostly implicated in regulating epigenome, majorly DNA methylation, in cardiovascular systems. However, its role in regulating histone methylation in cancers, is still under exploration. Herein, our study emphasizes the role of intracellular ROS on regulating epigenome, especially histone methylation or induction of repressive histone marks. Finally, as we know cancer is a developmental disease, we have established the role of one of the key signalling molecule-YAP, which is known to be involved in drug resistance, but from an epigenomic context. We observed that the upstream negative regulator of YAP- LATS is down-regulated through repressive histone methylation leading to increased YAP activity under drug stress. Moreover, beyond its conventional function, few studies show that YAP can work by

interacting with distal enhancers; however, its role in collaboration with histone repressive marks is not well established. Our study provides insights into how YAP can associate with EZH2 and can putatively regulate a repressive epigenome under drug stress facilitating tumor cell survival. Finally, we propose the use of combination therapy (Cisplatin plus epidrug/YAP inhibitor) as an alternative regime, which can be efficacious yet lower toxicities associated with current doses of parental drug used in combination.

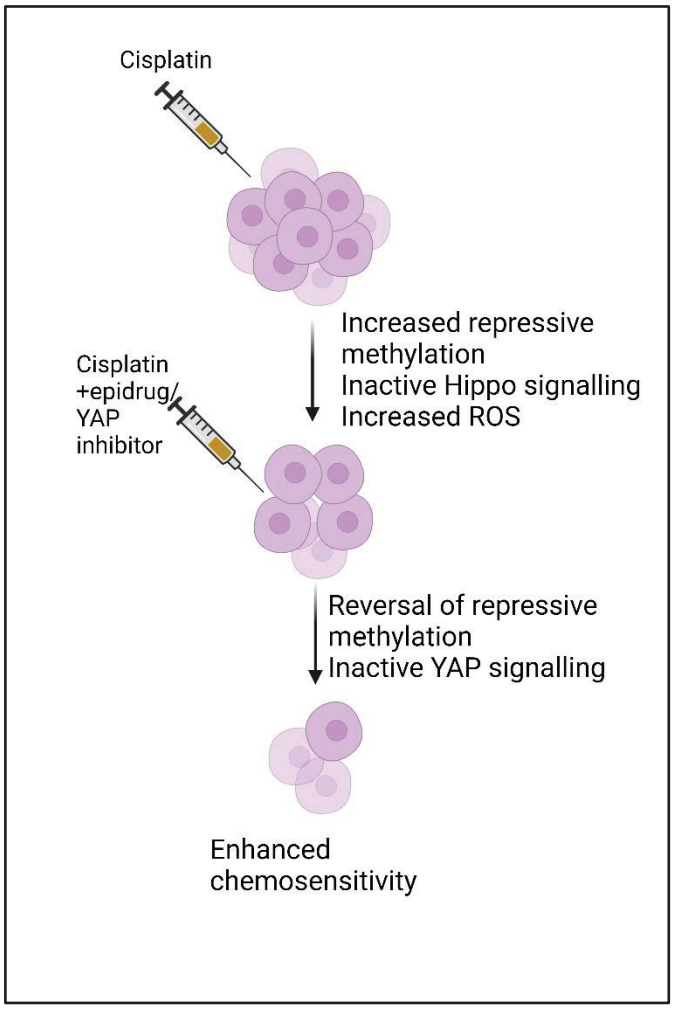


Fig.4.1 Schematic diagram highlighting major findings from the thesis

4.2 Limitations and Future perspectives:

1) This study exclusively analyses the histone methylation marks, however, a particular cancer may foster a combination of epigenetic modifications resulting in drug tolerance and subsequent relapse. A more complete picture of epigenomic landscape post-drug exposure is remaining.
2) The current study is restricted to *in-vitro* analyses; however, further *in-vivo* experimentations and clinical sample correlation can validate the proposed treatment option.
3) The type of drug, dose and time may also influence the dynamicity of these epigenetic modifications. The study could further be extended to look for similar or differential responses with other first-line chemotherapeutic drugs to create a database of epigenomic changes.
4) Finally, alongside efficacy, the toxicity profile of cisplatin with epidrugs or YAP inhibitor can be analysed and compared with existing drug regimen.

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Appendix I

A.1 List of Publications:

1. "Cisplatin-induced oxidative stress promotes YAP1-mediated epigenomic alterations facilitating the survival of Osteosarcoma cells." **Daiya A**, Chowdhury R, Chowdhury S, Mukherjee S (*Submitted to ACS Chemical Biology, Manuscript ID: cb-2024-00283r*).

2. "Transcriptomic analysis reveals differential adaptation of colorectal cancer cells to low and acute doses of cisplatin." Saini H; Dave R; Chatterjee S; Mandloi A; Sharma H; **Daiya A**; Mukherjee S; Chowdhury R and Chowdhury S. Gene; May 15; 2023

3. "A New Aggregation Induced Emission Active Halochromic White Light Emissive Molecule: Combined Experimental and Theoretical Study." Chaudhary J, Mittal V, Mishra S, **Daiya A**, Chowdhury R,* Laskar IR,* and Roy RK*. Journal of Phy. 202 Chemistry. May 2020

4. "Epigenetic adaptations in drug-tolerant tumour cells." Mani N, **Daiya A**, Mukherjee S, Chowdhury R and Chowdhury S. Advances in Cancer Research; Book Series; Elsevier; Nov 16; 2022 16; 2022 Nov 16; 2022 **(*Book chapter*)**

A.2 List of conferences/workshops attended:

1. **Ankita Daiya**, Rajdeep Chowdhury, Shibasish Chowdhury, Sudeshna Mukherjee. "ROS-H3K27me3 axis modulates expression of YAP to determine cellular fate of OS cell" at the **42nd Annual Conference of the Indian Association for Cancer Research** organized by ACTREC, Navi Mumbai, India. (***Poster presentation)***.
2. **International Conference-Life Science Research & its Interface with Engineering and Allied Sciences (LSRIEAS)**, Organizer, 2018, BITS Pilani, Pilani Campus, India.
3. A 2-day DBT/Wellcome Trust India Alliance Workshop on Science Communication, 2022 (Virtual)
4. DBT-BIRAC Workshop on Bio entrepreneurship and Intellectual Property & Technology Management in Life Sciences, 2023, BITS Pilani, Pilani Campus, India
5. Attended **Regional Young Investigator Meet on Life Sciences & Interdisciplinary Research - 2024** at BITS Pilani, Pilani Campus jointly organized by IndiaBiocience, BITS Pilani, IIT Jodhpur and CURAJ, Rajasthan.

Appendix II

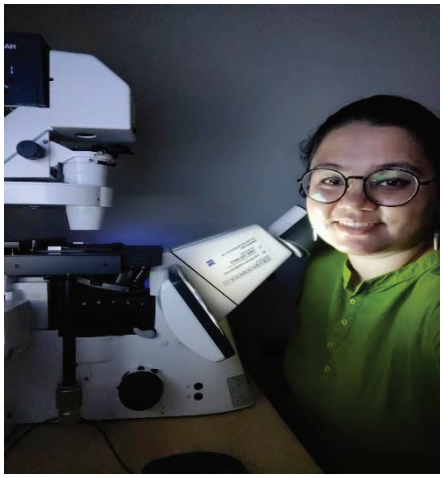
A.II.1 A brief biography of the Supervisor:

Sudeshna Mukherjee Chowdhury is currently working as Associate Professor, Department of Biological sciences, BITS Pilani, Pilani campus, Rajasthan. She did her Ph.D. from Chittaranjan National Cancer Institute (CNCI, Kolkata) under Jadavpur University. Her graduate research was primarily focused on elucidating the molecular mechanisms to carcinogenesis with special emphasis on cell cycle dys-regulation and chromosomal aberrations on embryonic fibroblasts. After her graduation she moved on to USA where she did her post-doctoral research from Department of Biochemistry, Tufts School of Medicine, Boston, USA. The research was focussed on Polyoma Middle T Antigen mediated signalling in cancer. She joined BITS-Pilani in July, 2017 with DST-SERB Young Scientist Grant. Post completion of that project she got a regular position as an Assistant Professor. Her expertise is in the field of cancer biology specifically epithelial to mesenchymal transition. She is interested in studying role of cytokines in tumor microenvironment and their cross-talk in EMT and cancer progression. She has received projects from various government funding authorities like - (i) SERB, Department of Science and Technology (SERB-DST) (ii) BITS, Research Initiation Grant (iii) ICMR (iv) BITS, Additional Competitive Research Grant (v) CSIR, Council of Scientific and Industrial Research as well. Findings from her works have been published in more than 30 reputed international scientific journals. At present she is guiding 4 Ph.D. students and have guided more than 10 M. Tech. Bio-Sciences students for the fulfilment of their dissertation.

A.II.2 A brief biography of the Co-Supervisor:

Prof. Shibasish Chowdhury obtained a master's degree in physical chemistry from Calcutta University. Then, he shifted to biophysics and obtained a Ph.D. degree from Molecular Biophysics Unit (MBU) at the Indian Institute of Science, Bangalore on "Computer modelling studies on G-rich unusual DNA structure". Subsequently, entered into the protein folding field and worked as a postdoctoral research fellow in the Department of Chemistry and Biochemistry, University of Delaware, the USA. Currently, Prof. Chowdhury is working as a Professor in the Department of Biological Sciences, Birla Institute of Technology and Science, Pilani. His major area of interest lies in elucidating complex relationships among sequence-structure-function and analysis of bio-molecular structures using model building and computational techniques. His group is involved in decoding inherent molecular signals within miRNA, which plays a crucial role in the specificity of target binding and gene silencing. He is involved in analyzing the molecular basis of different biological processes like drug resistance, molecular recognition, and evolution.

A.II.3 A brief biography of the candidate:



Ms. Ankita Daiya graduated in Biological Sciences from Birla Institute of Technology & Science, Pilani Campus, Rajasthan in 2016. She had her formal research training from one of the premier institutes in India-Advanced Centre for Treatment, Research and Education in Cancer, TMC, Mumbai as a project trainee in 2015, where she worked on identifying the binding partners of Gamma H2A.X in radio-resistant Glioblastoma. In the year 2018, she joined under Prof. Sudeshna Mukherjee Chowdhury, BITS-Pilani, Pilani Campus to pursue her doctoral research work as CSIR fellow. Her area of interest is cancer epigenetics. She has presented poster in one international conference and has published 2 research papers and 1 book chapter. Along with research, she was also involved in teaching and has taken courses for First degree and Higher degree students in BITS Pilani, Pilani Campus.