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Characterising the Role of OVOL1 in Cell Growth Regulation

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Abstract

The placenta supports the exchange of nutrients and gases between mother and fetus. Trophoblasts are the parenchymal cells of the placenta and perform the vast majority of its functions. There are different types of trophoblasts derived from stem cells called cytotrophoblasts (CTs). The balance between CT proliferation and differentiation is important for placental development. OVO-like 1 (OVOL1) is a transcription factor expressed in many epithelial lineages undergoing differentiation, including human differentiating CTs. The molecular mechanisms through which OVOL1 represses proliferation and/or promotes differentiation are unknown. We hypothesize that OVOL1 interacts with specific HDACs to repress CT proliferation. Ectopically expressing OVOL1 in wild-type yeast caused a significant growth defect, this defect was rescued by deleting class II HDACs. Ectopically expressing OVOL1 in human CT cell-line (BeWo) caused a significant increase in expression of *ERVFRD1*, a gene associated with cytotrophoblast differentiation, indicating that expression of OVOL1 is sufficient to trigger upregulation of at least a subset of genes that regulate CT proliferation and differentiation. Together, our findings demonstrate that OVOL1 can repress cell proliferation in yeast, a feature requiring specific HDACs, and is sufficient to at least prime CT differentiation. The combination of yeast and mammalian models provides a new experimental platform to better characterize OVOL1 function in repressing CT differentiation, providing new insights into placental development and potential therapeutics for placenta-associated diseases.

Keywords

OVOL1, HDACs, yeast, histones, BeWo, trophoblasts, cytotrophoblasts, syncytiotrophoblasts, differentiation, proliferation.

Summary for Lay Audience

During pregnancy, the placenta forms to facilitate the exchange of gases and nutrients between the mother and baby. When abnormal placental development occurs, complications can arise leading to serious harm to both the mother and baby. During the initial stages of pregnancy, important steps have to take place to ensure proper placental development. Formation of a specific placental cell-type that controls nutrient and gas exchange is regulated by a protein known as OVO-like 1 (OVOL1). However, there is very little known about how OVOL1 functions to form this important cell-type. Therefore, my goal is to explore the underlying mechanisms by which OVOL1 acts and how it works to trigger placental cells to undergo specialization and form different cell types. Therefore, my main aim is to explore the underlying mechanisms by which OVOL1 acts. I will be using yeast as a model organism to understand how OVOL1 affects gene expression. Yeast are single-celled eukaryotic organisms that share many homologous genes with humans, and allow us to conduct rapid, controlled experiments to identify how OVOL1 affects gene regulation. I hope to identify gene and protein targets that can act as future therapeutic targets to address placental maldevelopment. Future work will identify factors that affect OVOL1 function in yeast and translate our findings into mammalian models. My findings will provide insight into how the placental forms, which will help us better understand the causes of various pregnancy complications.

Co-Authorship Statement

I performed all the following experiments with the exception to the growth assays in Fig 1.1, which were conducted by Hazel Dhaliwal.

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Chapter 1

1 Literature Review

1.1 Pregnancy Complications

According to Statistics Canada, in 2018, one of the main leading causes of infant and maternal mortality and morbidity is maternal complications and diseases arising during pregnancy. Complications during pregnancy can include pregnancy loss, stillbirth, and hypertensive disorders[1]. 14% of maternal deaths are attributed to hypertensive disorders[2]. When patients are diagnosed with chronic hypertension, they have a three-fold increased risk of developing preeclampsia (PE) [3]. PE is a condition characterized by hypertension and protein in the urine (proteinuria) and affects close to 8% of pregnancies[4]. If serious cases are not immediately treated, it can lead to severe complications during pregnancy, delivery and postpartum. Over the last two decades, Auger et al. [5] found that the rate of PE in Canada increased from 26 to 50 per 1000 deliveries, which was accompanied with more intensive medical treatments. Furthermore, post-delivery, mothers diagnosed with PE suffered complications such as increased blood-brain barrier permeability, inflammation, hypertension, type 2 diabetes, acute and chronic vascular damage [6–9]. Siepmann et al. [10] found that there was a significant change in brain matter in women with PE. Specifically, white matter volume increased while gray matter volume decreased, and these structural impairments continued with time post-delivery. Removal of the placenta, which terminates the pregnancy, remains the only viable treatment option for serious PE cases; however, if this occurs prior to 37 weeks, severe neonatal complications associated with prematurity can arise.

PE not only affects mothers, but it can also directly and indirectly cause complications in the newborn. For example, PE is commonly associated with a serious prenatal complication called intrauterine growth restriction (IUGR). IUGR can also occur independently of preeclampsia. IUGR is a condition whereby the fetus is unable to reach its full growth potential due to genetic or environmental causes [11]. Babies diagnosed with IUGR are more likely to be associated with pre-term onset of PE[12], and have poor long term health outcomes. Crispi et al.[13] found that heart development in IUGR babies was

disrupted. Babies had thicker cardiac walls leading to decreased stroke volume and increased heart rate, all of which may increase risk of developing cardiovascular complications later in life[7,13–15].

PE and IUGR are often associated with poor development of the placenta. The placenta is a vital organ necessary for maintaining a healthy pregnancy. Korteweg et al. [16] investigated the causes behind intrauterine fetal death after 20 weeks of pregnancy, and found close to 67% out of 750 pregnancies were related to placental pathologies. Similarly, Horn et al. [17] discovered that almost 60% of stillborn deliveries were associated with utero-placental pathologies. Placental causes of death have been found in up to 65% of perinatal mortality cases[16]. Pregnancies diagnosed with IUGR have smaller placental volume with increased uterine vascular resistance [18]. In addition, Ray et al.[12] found an increase in placental insufficiency and vascular lesions with pregnancies complicated with IUGR. Given the importance of proper placental formation for healthy fetal development and pregnancy success, broadening our understanding behind pathways, factors and mechanisms that regulate placental development is important to help researchers develop more effective management strategies and therapies to reduce the risks and outcomes associated with pregnancy diseases.

1.2 Regulation of Placental Development

The placenta is a vital organ that develops during pregnancy. This transient organ regulates fetal growth by providing the growing embryo with adequate nutrients and oxygen, removing metabolic waste products, acting as a barrier against xenobiotics found in maternal circulation, and releasing hormones into both the maternal and fetal circulation to regulate fetal growth and placental function throughout gestation [19,20]. Understanding how it forms will help us discern what goes wrong during conditions such as PE and IUGR.

Formation of the placenta starts early during gestation. After the oocyte has been fertilized, the resulting zygote undergoes multiple mitotic divisions to form a hollow spherical structure known as the blastocyst. The blastocyst is made up of two important cellular structures: the outer trophectoderm layer and an inner cell mass. The trophectoderm gives rise to all trophoblast lineages that form the placenta. The inner cell mass, on the other hand, develops into all other structures including the yolk sac and embryo proper.

Approximately 7-8 days after fertilization, three important steps occur for the blastocyst to implant into the endometrial wall. First, the embryonic pole of the blastocyst made up of polar trophectoderm cells attaches to the uterine surface epithelium. Second, trophectoderm cells express many adhesion and regulatory factors such as integrins to allow for blastocyst adhesion. Lastly, a primitive syncytium that differentiates from cytotrophoblasts in the polar trophectoderm releases enzymes that break the endometrial wall and helps the blastocyst burrow deep into the decidua. Stem cells in the trophectoderm differentiate into cytotrophoblasts, which proliferate extensively to form primary villi. Cytotrophoblasts further differentiate into two cell lineages: extravillous cytotrophoblasts and villous cytotrophoblasts. Extravillous cytotrophoblasts form at the tips of the villi and differentiate into highly invasive cells responsible for transforming maternal tissue and remodeling uterine arteries, thereby improving blood flow to the placenta. Villous cytotrophoblasts serve as the progenitor cells of the placenta throughout pregnancy. These cells are responsible for continuous replenishment of a unique multinucleated lineage called syncytiotrophoblast that lines the

placental exchange surface and forms the barrier separating maternal and fetal blood[21–26], (Fig 1.1). Expression of syncytin genes like *ERVW-1*/ syncytin-1, *ERVFRD-1*/syncytin-2, *ERVV-1* and *ERVV-2* is important to induce fusion of cytotrophoblasts into the syncytiotrophoblast. Syncytin-1 and syncytin-2 are retroviral proteins only found in the placenta and are important for cell fusion.

Fusion is regulated by a number of processes that act similarly in vesicle trafficking and myocyte development. When fusion is induced, caspase 8 and 10 are activated which act to deactivate flippases/translocase, this indirectly causes a change in which change the location of negatively charged phospholipids known as phosphatidylserines from the inner to the outer leaflet of the plasma membrane[27,28]. In addition, proteases are also activated to remodel the cytoskeleton proteins and allow cells to come together and maintain the syncytiotrophoblast layer[28]. Growth factors, such as epidermal growth factor (EGF), are released by both the maternal and fetal environments[29,30]. EGF leads to the release of human chorionic gonadotropin (hCG) from syncytiotrophoblast and acts in a paracrine and autocrine fashion to induce cell fusion. They activate downstream signalling molecules such as PKA to increase expression of genes expressing syncytial proteins, syncytin 1 and syncytin 2[31,32]. Syncytin proteins are retroviral proteins that bind to amino acid transporters (ASCT1 and ASCT2) on the surface of plasma membrane to help cells fuse[26,33]. Other proteins are expressed during fusion to aid in membrane binding, like a disintegrin and a metalloproteinase domain (ADAM) protein family which contain fusion peptides[34].

In addition to forming the key barrier separating maternal and fetal blood, the syncytiotrophoblast acts as an immunological barrier and produces hormones vital for pregnancy. For instance, a characteristic of syncytiotrophoblast formation is an increase in expression of transcripts important for hormonogenesis, such as *CGA* and *CGB*, genes encoding the α and β subunits of hCG[35].

The placental exchange surface is arranged in finger like projections to allow for increase surface area and transfer of nutrient and oxygen. These projections are called chorionic villi which protrude into cavities, known as intervillous space, filled with maternal blood. The barrier is lined by syncytiotrophoblast, which faces the maternal circulation, and underlying cytotrophoblasts which line the basement membrane [19,20].

Importantly, cytotrophoblasts coordinately proliferate to maintain their stem cell population, or differentiate (fuse) to maintain syncytiotrophoblast integrity [20]. Differentiation of cytotrophoblasts is crucial for syncytiotrophoblast to expand and replenish, ensuring proper function of the exchange surface throughout pregnancy [19,20,36,37]. Fig 1.2 briefly summarizes the life cycle of cytotrophoblasts as they under proliferation and differentiation. Abnormal cytotrophoblast proliferation or differentiation can disrupt the integrity of the placental exchange surface and result in placental pathologies, such as PE and IUGR, causing serious harm to mother and baby[19,22,38–40].

Reconstitution of the syncytiotrophoblast is critical, since syncytiotrophoblast is continuously dying and being shed into maternal blood. As the nuclei within the syncytiotrophoblast age throughout gestation, they clump together at the surface of the villous tree to form groups of aged nuclei known as syncytial knots, which become apoptotic, and are shed into the maternal circulation [41]. Disturbances to the process of cytotrophoblast turnover may impair nutrient and gas exchange across the placental barrier. Studies looking at placental tissues have revealed pathological changes of preeclamptic and IUGR placentas compared to normal term placentas. Ezeigwe et al. [42] found that there was increase in cytotrophoblast proliferation. Arnholdt et al.[43] also found that this was accompanied by impaired syncytiotrophoblast formation when compared to normal term placentas. Preeclamptic placentas had elevated expression of Ki-67 (a proliferation marker) [44], increased activation of the ERK1/2 pathway (which promotes cellular proliferation)[45], and increased rate of apoptosis[46]. Furthermore, in PE and IUGR placentas, there is an increased level of syncytial knots and apoptosis [47]. The increase in apoptotic shedding leads to the release of cell-free fetal DNA and protein into the maternal circulation, resulting in inflammation. This process induces endothelial dysfunction, which causes activation of the maternal immune response and leads to maternal complications[41,48]. Dysregulation of villus trophoblast proliferation, differentiation and turnover may be one possible explanation underlying inadequate exchange of nutrients and aberrant placental development in IUGR and PE.

The regulation of cytotrophoblast proliferation and differentiation is not well understood. The placenta is vulnerable to changes in the external (xenobiotics exposure) and internal (maternal immune/inflammatory responses) environment, which may change

expression of critical genes involved in cell proliferation and differentiation and predispose to poor placental development in PE and IUGR [41,49]. In order for the placenta to respond to changes in its environment, cytotrophoblasts alter transcription of genes in part through epigenetic regulation [19,50]. Some studies report an increase in histone acetylation in placental tissues from IUGR pregnancies, possibly because increased acetylation alters the transcription profiles of genes related to normal placental development, syncytiotrophoblast function and cytotrophoblast differentiation [51,52]. The regulation of genes promoting proliferation in trophoblasts is not well understood and represents a major obstacle for understanding the underlying mechanisms governing placental development. Our lab previously discovered that OVO-like 1 (OVOL1) is highly upregulated during syncytiotrophoblast formation, and is critical for promoting cytotrophoblast differentiation [35]. Little is known about the underlying mechanisms of OVOL1's role in regulating trophoblast proliferation and differentiation, and thus will be the focus of my thesis.

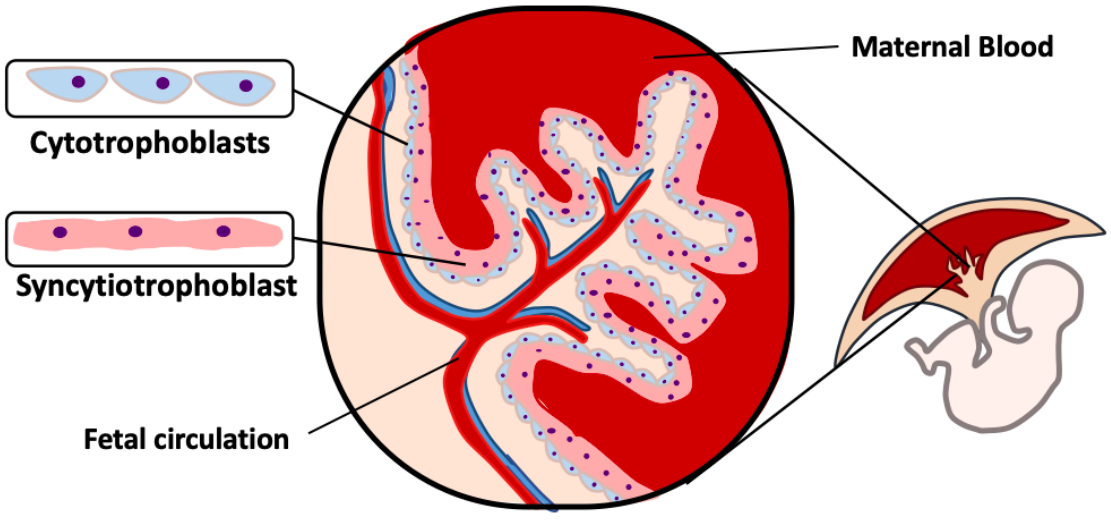

Figure 1.1 Features of the chorionic villi that make up the maternal-fetal barrier. The inner cytotrophoblast layer (blue cells) act as stem cells that undergo differentiation and fusion to form the outer syncytiotrophoblast (pink cells). The syncytium is in direct contact with the maternal blood which fills up the intervillous space.

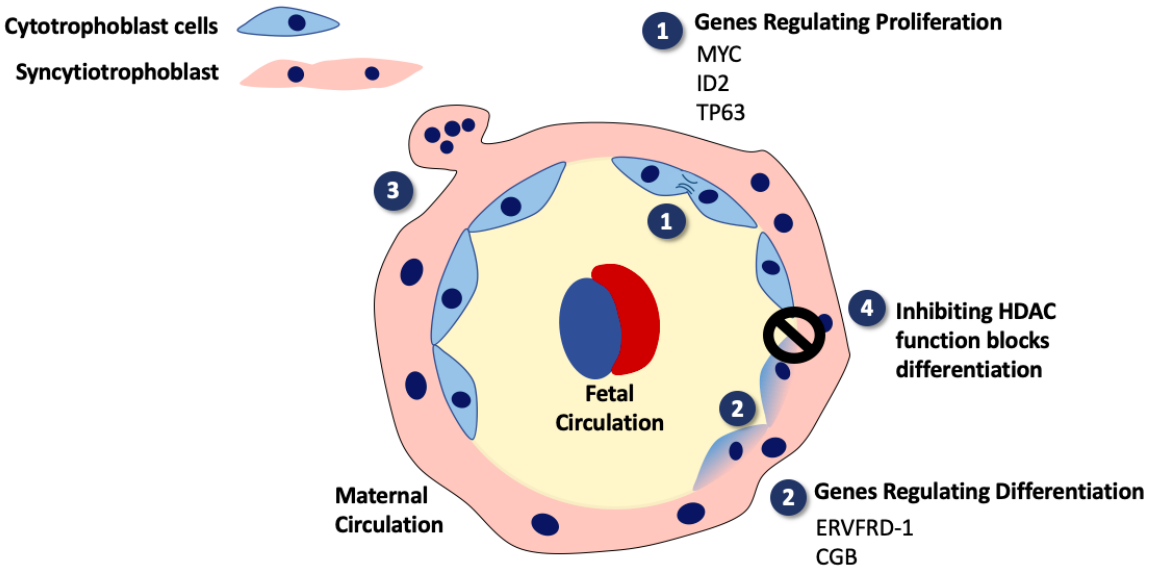

Figure 1.2 Summary of factors involved in trophoblast proliferation and differentiation.

Proper turnover of placental trophoblast cells is necessary to maintain structure integrity of the placental-maternal barrier. Depicted in this figure is a cross section of a chorionic villus. Cytotrophoblasts can proliferate and maintain their stem cell population by activating genes encoding proteins involved in fusion and hormonogenesis (1). In order for differentiation to occur, the stem cell-associated genes are repressed, and genes that promote fusion (ERVFRD-1) and hormonogenesis (CGB) are expressed to allow for cells to form the overlaying syncytiotrophoblast (2). Lastly, aged nuclei cluster together at the surface known as syncytial knots, which will be expelled into the maternal circulation (3). (4) Functional HDACs are required for proper differentiation of cytotrophoblasts.

1.3 OVO-Like Family of Proteins

OVOL1 is part of a conserved family of genes that encode C_2H_2 zinc finger "OVO-like" transcription factors. Currently there are 3 OVO-like genes, OVOL1, OVOL2 and OVOL3 [53–55]. OVOL proteins were first characterized in *Drosophila,* they were responsible for proper differentiation of the germline and oogenesis [56]. They are also expressed in epithelial and germ cell development in many organisms such as *C. elegans*, *D. melanogaster* and *M. musculus* [56–59]. OVOL1 plays an important role in epithelial cell development across organisms and could be implicated in human diseases when the balance between epithelial proliferation and differentiation is dysregulated.

Furue et al.[60] conducted a meta-analysis genome wide association study and found that OVOL1 was strongly associated with atopic dermatitis, a common skin disorder. They discovered that OVOL1 works downstream of inflammatory markers in the skin, and when it becomes inactivated, leads to skin barrier dysfunction which leads to inflammation[60]. Jiang et al.[61] also found that OVOL1 was implicated in nasopharyngeal carcinoma by repressing proteins involved in angiogenesis and tumorigenesis. Similarly, work done by Tsuji et al. [62] revealed that OVOL1 controls expression of skin barrier proteins in human keratinocytes[62]. OVOL1 is upregulated in cutaneous squamous cell carcinoma (CSCC) and decreases in expression as cells shift into a more invasive state[63]. OVOL1 works by repressing c-Myc expression to prevent CSCC from becoming invasive[63]. OVOL1 regulates interfollicular epidermal development by binding to the promoter of c-Myc, repressing its transcription and activating differentiation[64].Similarly, OVOL2 also represses c-Myc expression thereby inhibiting terminal differentiation in human keratinocytes [65]. OVOL1 also works with OVOL2 in human hair bulb cell proliferation and differentiation[63]. Ito et al.[54] and Teng et al.[66] discovered that OVOL1 was able to downregulate OVOL2 expression in human keratinocytes, suggesting OVOL2 to be a downstream target of OVOL1. They also showed that OVOL1 is critical for keratinocyte terminal differentiation while OVOL2 suppresses differentiation. OVOL1 expression is important in other epithelial cell lineages, upregulation of OVOL1 inhibits oral squamous cell carcinoma proliferation by repressing ZEB1 (a transcription factor) to prevent metastasis[67].

Ablation of OVOL1 in mice results in skin progenitor cells proliferating abnormally leading to aberrant skin development and mammary gland branching [57,68–70]. Somatic cells of mouse embryonic fibroblast can undergo reprogramming to become induced pluripotent stem cells (iPSCs); however, some cells fail to transition, this is where OVOL1 was discovered to repress cellular proliferation and expansion[71]. Keratinocytes derived from OVOL1 ablated mice showed increase cell proliferation, and mice showed increase skin permeability when either OVOL1 or OVOL1 and OVOL2 were knocked out[66]. OVOL1 also plays a role in osteogenesis, Min et al.[72] found that by ectopically expressing OVOL1 in mice, it enhanced expression of osteogenic genes which allowed for osteoblast differentiation in mice and an increase in expression of osteoblast differentiation markers such as Id2. OVOL1 and OVOL2 work together to inhibit epithelial-to-mesenchymal transition (EMT) in certain epithelial tissues and cancer cell lines [69,73]. In contrast, in mice prostate cancer cells, OVOL1 and OVOL2 induce a mesenchymal to epithelial transition (MET) [73]. Murata et al. [74] found that ZEB1 expression was higher in tumors with low OVOL2 expression. Both OVOL1 and OVOL2 affect ZEB1 expression, OVOL2 directly downregulates ZEB1 expression while OVOL1 indirectly decreases ZEB1 transcript levels[74]. This regulation is important because it is believed to prevent the development of CSCC[74]. Lastly, OVOL1 promotes pancreatic stem cell differentiation into endocrine cells[75]. OVOL1 protein levels increases in pancreatic differentiated cells, which suggests that OVOL1 promotes cells to achieve a more differentiated state in pancreatic tissues of mice[75].

The OVOL family of proteins play important roles in epithelial development. Mouse OVOL2 is important for cranial tube development, and deletion of OVOL2 in mice causes severe defects during cranial neural tube formation which leads to embryos mortality. OVOL2 induces neuroectodermal differentiate, and is necessary for proper brain, neural crest, gut tube and heart development in mice[76]. It is also critical in testis development, brain, and epithelial development in the skin and intestine of adult mice[77]. However, postnatal germ cell development and spermatogenesis is regulated by OVOL1 and not OVOL2 in mice[78]. Unezaki et al. [58] found that OVOL2 was important during vascular angiogenesis during early embryogenesis, and trophoblast differentiation; embryos abnormally develop and are small in the absence of OVOL2[58].

Interestingly, in different cell types, OVOL1 functions as a gene repressor, and in certain cases it repress genes downstream of Wnt/ β-catenin and BMP/TGF-β signaling pathways[23,77,79,80]. Wnt signaling is also important in placental development in mice and humans [80–82]. Wang et al.[83] found that expression levels of signalling molecules like Wnt and β-catenin were upregulated in PE.

OVOL1 is expressed in skin, kidney and male germinal epithelium [57], and is able to repress *OVOL1* expression and downstream targets by binding to a core hexamer sequence, CCGTTA, within the promoter regions of genes such as c-Myc and Id2 [64,84–86]. Nair et al.[84] found that OVOL1 binds to its own promoter by competing with c-Myb (proto-oncoprotein) and represses its own transcription. OVOL1 transcript levels are restored in the absence of a functional OVOL1 protein, and with increased expression of c-Myb. However, little is known about the role of OVOL1 during trophoblast proliferation and differentiation. Since OVOL1 acts to repress proto-oncoproteins and genes critical for cell proliferation, we postulate that OVOL1 may similarly control cytotrophoblast proliferation. Nair et al.[64] found that OVOL1 was important in mice interfollicular epidermal differentiation, in the absence of OVOL1 the progenitor cell layer in the epidermis of adult mice was expanded. Without OVOL1, the epidermal progenitor cells fail to stop proliferating, even in response of extrinsic growth inhibitory signals like LiCl, TGF-beta and Ca^{2+} .

DNA microarray analysis conducted by Renaud et al. [35] revealed that *OVOL1* was the most highly unregulated transcript encoding a transcription factor in differentiating BeWo cells – a choriocarcinoma cell line that is commonly used as a model of cytotrophoblast differentiation. Knockdown of OVOL1 impaired the capacity of these cells to fuse. When BeWo cells were induced to differentiate using 8-Bromo-cAMP, there was a dose dependent increase in *OVOL1* transcript levels[35]. Furthermore, *OVOL1* expression is high when primary cytotrophoblasts spontaneously fuse; however, OVOL1 levels decrease once syncytiotrophoblast forms [35]. Knockdown of OVOL1 in BeWo cells correlated with a decrease in the ability of these cells to fuse. They also performed RNA-seq analysis and found that genes encoding proteins associated with syncytiotrophoblast hormonogenesis such as *CGA, CGB, HSD3B1*, and *CYP19A1* were downregulated in OVOL1-deficient cells. Syncytin genes (*ERVW-1/ syncytin-1*, *ERVFRD-1/syncytin-2*,

ERVV-1 and *ERVV-2*) are endogenous retroviral derived genes which drive cell fusion. In the absence of OVOL1, there was a markedly decreased expression of these syncytin genes [35].

How OVOL1 is able to control cytotrophoblast differentiation is still unclear. OVOL1 directly binds upstream of several genes implicated in cytotrophoblast proliferation – *MYC, ID1, TP63* and *ASCL2* – by binding to CCGTTA sequences located in the proximal promoter regions of those genes, suggesting that OVOL1 is able to regulate expression of genes important for proliferation[35]. Further, OVOL1 does not seem to activate fusogenic genes such as *ERVFRD-1*[35]. Whether OVOL1 recruits co-repressors or forms a complex of transcription factors to downregulate genes involved in proliferation is unclear, and understanding the underlying mechanisms governing OVOL1's action will be the focus of this thesis. To delineate the importance of specific domains of OVOL1 in causing growth arrest in yeast, we used two mutated versions of *OVOL1*. Nair et al.[84] discovered that mutating the cysteines to alanines in the first three zinc finger motifs of OVOL1 disrupted OVOL1-DNA binding and proper zinc finger formation inhibited the capacity of OVOL1 to exert transcriptional repression. Thus, we wanted to use a similar strategy to determine whether the decrease in yeast growth is due to OVOL1 binding to DNA through its zinc finger domains. Three different forms of OVOL1 will be used to study its function in cell growth and proliferation summarized in Fig 1.3: wild type OVOL1, SNAG-deleted OVOL1 (Δ15 OVOL1), and zinc-finger mutated OVOL1 (ZnFC2A).

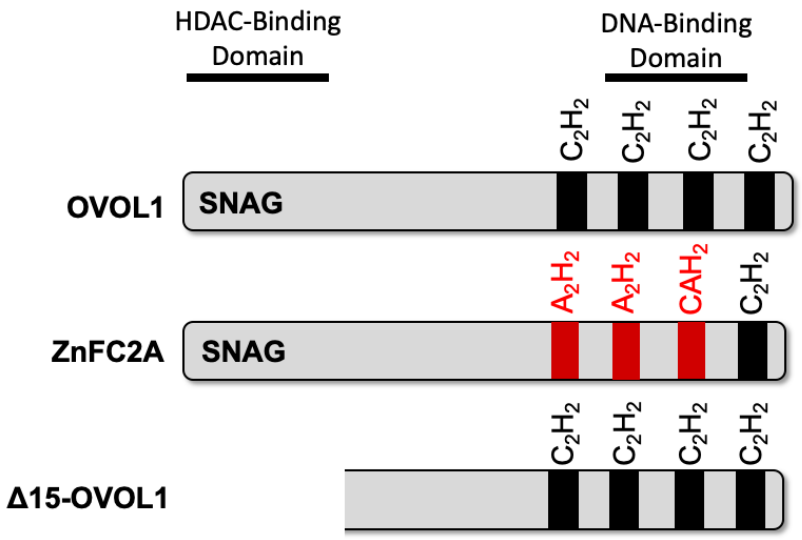

Figure 1.3 OVOL1 protein structures and mutations used in this thesis.
OVOL1 contains two conserved domains, the SNAG domain responsible for binding onto histone deacetylase proteins, and four zinc-finger domains responsible for DNA binding. ZnFC2A- OVOL1 mutation is produced by mutating the first two zinc finger binding domains from cysteines to alanines, and the addition of an alanine in the third zinc finger region. Δ15-OVOL1 is produced by deleting the first 15 amino acids which includes the SNAG domain.

1.4 Histone Deacetylases and the Placenta

Epigenetic modifications are essential in regulating proliferation and differentiation of all stem cell lineages, and aberrant gene regulation caused by perturbations in epigenetic modifications has been documented in a number of diseases such as cancers, neurodegenerative diseases, and placental diseases [20,50,87–92]. Gene expression can be regulated using diverse mechanisms including DNA methylation, non-coding RNA or through post-translational modifications of histones [93,94]. Histones are crucial proteins responsible for packaging the long strands of DNA into a more condensed structure known as chromatin. Two copies of each histone proteins H2A, H2B, H3 and H4 assemble together to form an octameric complex to allow for the DNA to wrap around. Post-translational modifications can occur on histone N-terminal tails through methylation and acetylation to regulate the packaging density of DNA [90,95]. Histone posttranslational modifications include methylation, sumoylation, phosphorylation, and acetylation [95–97]. Our lab recently showed a key role for histone (de)acetylation during cytotrophoblast differentiation [98]. Therefore, our work focuses primarily on understanding how pathways that regulate acetylation affects cellular proliferation in placental development. Acetylation is a dynamic process regulated by both histone acetyltransferases (HATs) and histone deacetylases (HDAC). HATs and HDACs act in opposition to each other, HATs add acetyl groups to ε-lysine residues on the N-terminal tails of histones and non-histone proteins, while HDACs remove acetyl moieties [92,99]. Acetyl groups act to neutralize the positive charge on histone complexes, which allows the DNA to be in a more relaxed state. Conversely, HDACs remodel chromatin into a more condensed state, preventing transcription factors from accessing DNA thereby repressing gene expression[92,95,97,100–104]. Fig 1.4 provides a summary of how HDACs are able to remodel the chromatin. Generally, hyperacetylation of histones leads to gene expression, while hypoacetylation results in gene repression [101,102,105]. Transcriptional dysregulation can lead to abnormalities in cell proliferation and differentiation; aberrant recruitment of HDACs has been linked to cancer, neurodegenerative disorders, and pregnancy-related disorders such as PE and IUGR [36,87,101,106–109]. HDACs do not directly bind to DNA, and therefore must operate in multiprotein complexes to modify both

histones and proteins [110]. Multiprotein complexes acts as corepressors and include the interaction of HDACs with Sin3, nucleosome remodeling and histone deacetylation (NuRD) and CoREST [110–112]. HDACs also regulate acetylation on non-histone proteins, like p53 and α-tubulin, to regulate cellular functions such as RNA processing, cellular metabolism, and apoptosis [113–115].

HDACs fall into two protein families: NAD^+-dependent and Zn^{2+}-dependent. The Zn^{2+}-dependent HDACs are further subdivided into three classes (class I, II, and IV). The NAD^+-dependent HDACs comprise class III HDACs.[99,101,102,104,116]. HDACs are highly conserved among eukaryotes. For example, class I HDACs are closely related to the yeast histone deacetylase, RPD3; class II is linked to yeast HDA1; and class III linked to yeast SIR2 [101–104]. Some of these HDACs localize exclusively to the nucleus and others shuttle between the nucleus and the cytoplasm [101,102,104]. The interplay between function and localization of HDACs is important to regulate genes involved in cellular proliferation. HDAC inhibitors have been widely studied as therapeutic targets to treat proliferating cancer cells [117].

HDAC1 and a subunit of the NuRD complex binds to genes implicated in embryonic stem cell pluripotency, including Oct4 and Nanog, causing transcriptional repression of these genes [118]. Furthermore, HDAC inhibitors such as trichostatin A (TSA) have been used to delineate the roles of HDAC function in embryonic stem cell function[117,119]. Kidder and Palmer[118] conducted a transcriptome analysis mouse trophoblast cells treated with TSA revealed that HDAC1 was able to bind to stem-cell associated genes enriched in trophoblast cells such as Cdx2, Elf5, Eomes and Sox2 – all of which prevent trophoblast lineage differentiation. The blastocyst has two stem cell lineages, embryonic stem cells and trophoblast stem cells, and during implantation two molecules are released to allow for each cell population to self-renew. In mice trophectoderm development, HDAC1 binds to promoter regions of genes like LIF and FGF4, involved in the stimulating the inner cell mass to proliferate and in trophoblast self-renewal, respectively [118].

HDAC1 can interact with BRG1, a chromatin remodelling protein, to coregulate a number of genes involved in mouse embryonic stem cell development[120]. In addition, HDAC1 is able to bind to cell-cycle proteins such as cyclin-dependent kinases (such as

Cdk4), and cyclin-dependent kinase inhibitors (Cdkn1a/b), c-Myc, and p53 to regulate cell cycle progression and tumorigenesis in trophoblast cells[120].

Jaju et al.[98] found that treating cytotrophoblast cells with TSA reduced differentiation potential of BeWo trophoblasts. This was correlated with TSA-dosage dependent increase in acetylation of the following: histone 2B-lysine site 5 (H2BK5), histone 3-lysine site 27 (H3K27) and 14 (H3K14). These results suggest that deacetylation is an important mechanism involved in syncytialization. Interestingly, HDAC1 and HDAC2 were found to be critical in trophoblast cell fusion. HDAC1 and HDAC2 have similar and distinct biological functions. For example, there is no phenotypic changes in tissues such as smooth muscle, epidermis and cardiomyocytes when HDAC1 or 2 are ablated, suggesting that these HDACs can compensate for each other[121–123]. However, a double knockout of HDAC1 and 2 results in severe defects in these tissues[124]. Similar to other tissues, Jaju et al.[98] found that the knockdown of either HDAC1 or HDAC2 did not affect cytotrophoblast differentiation; however, upon ablating both, cytotrophoblasts ceased to differentiate.

As described thus far, HDAC function and regulation of gene expression is crucial for proper trophoblast proliferation and differentiation. Whether HDACs and OVOL1 function cooperatively to regulate cytotrophoblast cells is not known. Nair et al.[84] discovered that when overexpressing OVOL1 and HDAC1 in H293 cells, OVOL1 was able to bind to HDAC1 through its N-terminal SNAG transactivation domain. The SNAG region is a conserved region found in other zinc finger transcriptional repressors, such as Snail1 and Snail2, transcription factors involved in EMT induction[125]. Snail proteins are able to recruit chromatin remodelling complexes consisting of HDAC1 and HDAC2 [125,126]. These Snail-proteins interact with NCoR within the HDAC1 complex[125]. Deletion of the first 15-amino acids which includes the SNAG domain and mutation in the zinc finger region of OVOL1 disrupts the ability of OVOL1 to repress gene expression[84]. When Nair et al. [84] co-expressed OVOL1 and HDAC1, there was enhanced repression of *OVOL1*. In addition, OVOL1 decreases acetylation of histone 3 (H3) on the *OVOL1* promoter. When the OVOL1 zinc finger region is mutated or when OVOL1 displaces c-Myb binding, it causes a decrease in acetylation of H3, which strongly suggests that OVOL1 mediates transcriptional repression by deacetylating histones near target genes. It

is currently unclear whether there is a genetic interaction between OVOL1 and HDACs. To study the nature of the relationship between OVOL1 and HDACs in cellular proliferation, I will employ a combination of human trophoblast model and the budding yeast *Saccharomyces cerevisiae*, that recapitulates many of the features of the human transcriptional regulation, including histone acetylation.

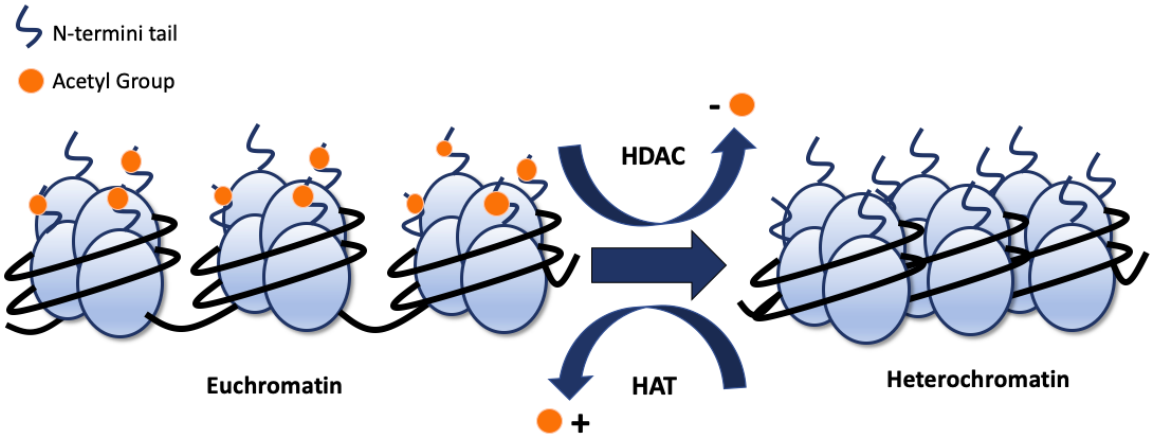

Figure 1.4 Histone posttranslational modifications alter chromatin structure. DNA wraps around an octamer of histone proteins that form a nucleosome. Histones have an N-terminal tail that is post-translationally modified to regulate gene expression. HDACs remove acetyl groups while HATs add acetyl groups to lysine on histone tails. The acetyl moiety acts to neutralize the positive charge on the histone proteins, which relaxes chromatin structure and allowing for transcription factor accessibility.

1.5 Yeast as Model Organism to Study Transcriptional Regulation

Studying transcriptional regulation, protein interaction and overexpression is difficult in mammalian cultures due to the complexity of its signalling pathways. Yeast *Saccharomyces cerevisiae* offer researchers a simple cellular model to study signaling pathways and protein interactions. It has a high degree of conserved cellular processes and mechanisms with higher level eukaryotes such as humans, including processes that regulate cell division, gene expression, cell death, metabolism, DNA repair protein folding and degradation [115,127–131]. Many tools exploring gene expression and conserved protein interaction networks were first developed and validated using a yeast model [132]. Yeast are easy to genetically manipulate, harvest and perform genome wide screens due to the availability of a plethora of gene deletion libraries [133,134].

The discovery of histone function and its protein interactions were first identified in yeast. There are two pairs of conserved genes encoding the four histone proteins that make up the nucleosome, two genes encoding H2A and H2B, and the other two encoding H3 and H4. Studies that used yeast as a model were first to demonstrate that a mutation in both *HTB1* and *HTB2*, encoding histone H2B, produce inviable cells[135]. Nucleosomes will compact the DNA into a chromatin structure that can be remodelled when histones are post-translationally modified. Early studies looking at gene repression was demonstrated in yeast looking at *PHO5* (acid phosphatase) promoter activity under high phosphate environment which would suppress PHO5 activity by altering histone expression levels [136]. When histone H4 expression was reduced, the *PHO5* promoter became activated. Studies on phosphate regulated genes like *PHO5*, *PHO2*, and *PHO4* helped establish our knowledge surrounding nucleosome function in remodelling chromatin and preventing transcription factors from accessing promoters [136]. Interaction networks established a conserved link between humans and yeast, and showed that chromatin structure is controlled by conserved factors [137–140]. Yeast proteomics and gene expression data has been applied to data on humans and other model organisms [141–145].

Given the complexity of epigenetic regulation, the yeast model provides us with a relatively small number of proteins implicated in epigenetic regulation. For instance, yeast only rely on histone modifications to change epigenetic states, which simplifies studying epigenetic markers[94]. Therefore, yeast make an excellent model organism to study the interplay between transcription factors, histone deacetylases and their effect on cell growth and proliferation. Much of our understanding of transcriptional regulation in eukaryotic cells comes from yeast studies. Many factors are highly conserved, such as the human and yeast TATA-box binding proteins (TBP), which share 80% identity and are functionally conserved. Either human or yeast TBP can be used for transcription by RNA polymerase II. Moorefield et al. [146] expressed human RNA polymerase I, encoded by the human *RRN3* gene, in yeast cells possessing a non-functional *RRN3* gene, and it was able to rescue a severe viability defect.

Human proteins involved in regulating cell cycle control are also conserved in yeast. Yeast only express one heat shock factor (HSF), which differs from humans, and the human HSF1 functionally complements the HSF found in yeast. Similar to the RRN3 experiment described above, heterologous expression of the human *HSF1* gene rescued a growth defect in yeast with HSF deletion [147]. Aberrant cell cycle control can lead to cancer and tumorigenesis. Both tumor and yeast cells share similar metabolic pathways. The RAS subfamily of proteins are highly conserved between humans and yeast, yeast express Ras1 and Ras2 proteins regulating the cAMP/PKA signalling pathway[148,149]. Yeast RasV119 protein which is homologous to human RasV12 (encoded by an oncogene) was heavily studied in yeast, and was discovered to increase mitochondrial metabolism which is important during tumorigenesis[148,149]. PI3K/AKT pathways is also heavily conserved in yeast, yeast encode SCH9 which is homologous to human Akt1 important in coordinating cell metabolism and the TOR signalling pathway[148–150]. When yeast are in a carbon rich media, they prefer to use a process known as fermentation instead of oxidative metabolism[151]. Glucose is able to repress oxidative metabolism in yeast, and this is similar in tumor cells. This process is regulated by human Akt and yeast Sch9 protein, all involved in TOR signalling pathway[151]..

Furthermore, heterologous expression of human genes implicated in cancer progression, such as *p53, BRCA1* and *BRCA2* in yeast helped pave our understanding of

not only tumorigenesis, but also cell cycle regulation, protein synthesis, autophagy, and proteasome activity – all pathways highly conserved in yeast [113,152,153]. Functional analysis of p53 mutations have led to the discovery of target genes in yeast, and were found to induce apoptotic-like cell death in yeast [153]. Tumor suppressor genes like *BRCA1* and *BRCA2* have also been successfully expressed and studied in yeast; expression of *BRCA1* causes cell growth arrest and phenotypic changes in yeast [153,154].

Yeast have been used extensively to investigate epigenetic regulation, chromatin remodeling and cellular proliferation. Importantly, HDAC protein sequences are highly conserved across eukaryotes including yeast (Table 1.1). Duffy et al [155] used the yeast acetylome to investigate the functional differences between different HDACs. They found that yeast HDACs that work in complexes, like Sin3-Rpd3 complex and HAD complex, have similar and distinct interaction profiles. There are 327 unique interactions found in the class II family of HDACs, where only 7% of those interactions were shared among each subunit and 55% were specific to each HDA1, HDA2, and HDA3 proteins. Interestingly, the HAD complex is important in peroxisome translation, but HDA2 protein is only involved in regulating peroxisome biogenesis. HDACs can also deacetylate non-histone proteins, and many human proteins that are acetylated have yeast homologs[156]. Acetylation can affect protein activity, stability, interaction and localization. In a yeast RPD3 deletion screen, Duffy et al. [155] found that 4 out of the 187 proteins tested had a defect in localization which were membrane proteins, ubiquitin domain-containing protein – UBX3, and receptors important in drug and ion transport.

Functional assays like genome wide screens and growth assays can be used in yeast to determine targets and effects of heterologous expressed genes in cell cycle regulation and growth. HDACs and OVOL1 are both implicated in cellular proliferation in cytotrophoblasts, and whether there is a genetic interaction between them is unknown. There is no known yeast homolog of human OVOL1, and this is important because it allows us to explore OVOL1 in a genome uncomplicated by other pathways involved in human cell proliferation. By using the highly conserved yeast genome, we can better delineate interaction networks of OVOL1.

Table 1.1 HDAC Classes Conserved between Yeast and Humans.

HDAC proteins are highly conserved across eukaryotic organisms and are grouped based on sequence similarities [99,102].

Class	*Saccharomyces cerevisiae*	*Homo sapiens*
I	Rpd3 Hos1 Hos2 Hos3	HDAC 1, 2, 3, 8
II	Hda1 Hda2 Hda3	HDAC4, 5, 6, 7, 9a, 9b, 9c, 10
III	Sir2 Hst1 Hst2 Hst3 Hst4	Sir1, 2, 3, 4, 5, 6, 7
IV	(Not found in yeast)	HDAC11 (has been characterized to have both class I and II functions)

1.6 Hypothesis

How Ovol1 is able to bind to DNA and regulate gene expression during cell division and differentiation is not well understood. Therefore, understanding the underlying mechanisms of gene regulation by OVOL1 will further elucidate components of placental biology as well as organ development. Recently, Bhattad *et al.* 2020 found that HDAC1 and HDAC2 are necessary for cytotrophoblast differentiation. **Therefore, I hypothesize that OVOL1-dependent regulation of gene expression requires HDACs to regulate cellular proliferation.**

1.7 Objectives

My project employs a combination of two cellular models: the budding yeast *Saccharomyces cerevisiae* and human BeWo cytotrophoblast-like cells. Due to the high level of conservation in cellular pathways and transcriptional regulation between yeast and humans, yeast is a valuable tool to study novel transcription factors and their role in gene regulation. Therefore, my primary objective is to study the effect of OVOL1 expression on cell growth in yeast and test the importance of HDACs for OVOL1-mediated effects.

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2 Characterizing the Role of OVOL1 in Cell Growth Regulation

2.1 Materials and Methods

2.1.1 Cell Strains and Media Conditions

Saccharomyces cerevisiae yeast strains used are summarized in Table 2.1. All strains used are derivatives of BY4742. Yeast cells are thawed from frozen glycerol stocks onto YPD (yeast extract peptone dextrose) plates for two days at 30°C. All yeast cells were cultured into selective synthetic complete media containing 2% w/v glucose and incubated at 30°C overnight. In order to induce expression of OVOL1, cells are washed twice in media containing 2% w/v galactose and resuspended at optical density (OD_{600nm}) 0.2 and incubated overnight at 30°C in galactose containing media.

Human BeWo trophoblast-like cells, which are derived from choriocarcinoma and are a well-established model of cytotrophoblast-like cells (ATCC CCL-98) were thawed from frozen stocks into DMEM: F12K media supplemented with 10% Fetal Bovine Serum (FBS) and 0.5% Penicillin/Streptomycin (P/S). Cells are passaged at 30% confluency into 24-well plates for transfections.

Table 2.1 List of Yeast Strains.

Yeast Strains	Genotype	Reference
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	1
hda1Δ	Same as BY4742 except *HDA1::KAN*	Deletion array
hda2Δ	Same as BY4742 except *HDA2::KAN*	
hda3Δ	Same as BY4742 except *HDA3::KAN*	
rpd3Δ	Same as BY4742 except *RPD3::KAN*	
hos1Δ	Same as BY4742 except *HOS1::KAN*	
hos2Δ	Same as BY4742 except *HOS2::KAN*	
hos3Δ	Same as BY4742 except *HOS3::KAN*	
sir2Δ	Same as BY4742 except *SIR2::KAN*	
hst1Δ	Same as BY4742 except *HST1::KAN*	
hst2Δ	Same as BY4742 except *HST2::KAN*	
hst3Δ	Same as BY4742 except *HST3::KAN*	
hst4Δ	Same as BY4742 except *HST4::KAN*	

2.1.2 Plasmid Construction

OVOL1, ZnFC2A, and Δ15-OVOL1 were synthetized by Thermo Fisher Scientific. Using polymerase chain reaction (PCR), OVOL1 fragments were generated and cloned into p415-gal1 and p416-gal1 plasmids using SpeI/SalI restriction enzymes. Plasmids were validated by sequencing them at Robarts Sequencing Facility (London ON). All plasmid constructs are listed in Table 2.2 for yeast plasmids and 2.3 for mammalian plasmids.

Table 2.2 List of Yeast Plasmids.

Plasmids	Vector Backbone	Resistance	Reference
GAL1-OVOL1	pRS415 (CEN/ARS)	Leu^+	This paper.
GAL1-ZnFC2A			
GAL1-Δ15OVOL1			
OVOL1-ymsfGFP			
GAL1-OVOL1	pRS416 (CymEN/ARS)	Ura+	
GAL1-ZnFC2A			
GAL1-Δ15OVOL1			

Table 2.3 Mammalian Plasmids.

Plasmid	Vector Backbone	Reference
pEF-GFP	pCAGEN	Addgene (#11154)
pEF-OVOL1		This paper.

2.1.3 Yeast Transformation

Plasmids were transformed into yeast strains (listed in Table 2.4) using a high-efficiency lithium acetate protocol described in Gietz et al.[157]. In brief, cells were cultured overnight, and cells at OD_{600nm} of 1.0 were pelleted at 3000 × g for one minute in a 1.5 mL Eppendorf tube. Cells were then washed and incubated for five minutes at room temperate using 1.5 mL of sterile 0.1 M Lithium Acetate (LiAc) in Tris and EDTA (TE) Buffer. Cells were then resuspended in 285 µL sterile 50% PEG, 2.5 µL plasmid and 10 µL boiled salmon sperm DNA. Cells were incubated at 30°C for 45 minutes. Following incubation, 43 µL of sterile DMSO was added to cells and heat shocked at 42°C for 15 minutes. Cells were then plated onto selection agar plates.

2.1.4 Growth Assays

Cells were grown in glucose overnight then subsequently washed and incubated in galactose media (refer to section 2.1.1). They were then serially diluted five times at OD_{600nm} 0.2 in a 96-well plate and spotted onto agar plates. Plates were incubated for two nights and imaged using a Bio-Rad Laboratories GelDoc System.

Liquid growth assays were conducted using the same cells that were spotted onto agar plates. Using a clear 96-well plate, diluted cells were replicated 3 times, and OD_{600nm} was measured every 15 minutes for 24 hours using a Biotek plate reader.

2.1.5 Fluorescent Microscopy

WT and *hda1Δ* cells expressing OVOL1ymsfGFP were induced in galactose media as described in 2.1.1. Cells were diluted 10X, transferred into a 6-well plate, and

imaged at room temperature using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) with a 20X objective lens and EGFP filter (488-509 nm). Images were analyzed using ImageJ software (https://imagej.nih.gov/ij/).

2.1.6 BeWo Trophoblast Cell Transfection

BeWo cells were split into 24-well plates at around 30% confluency. The following day, media were changed, and transfection was conducted according to JetPrime DNA transfection protocol (https://www.polyplus-transfection.com/products/jetprime/). Briefly, BeWo cells were transfected with plasmids found in Table 3. In brief, 1.5 µg of DNA was diluted into 200 µL JetPrime Buffer and vortexed for 10 seconds. 3 µL of JetPrime reagent was added, vortexed for one second, and incubated at room temperature for 10 minutes. 200 µL of the mixture was added into each well in a drop-wise manner. Two sets of controls were used, one well had a regular media change (non-transfected), while the other control was transfected with the vector backbone. BeWo cells were also induced to differentiate for 48 hours (hrs) using 10 µL/ng of cell-permeable derivative of 3′, 5′-cyclic adenosine monophosphate (cAMP).

2.1.7 Quantitative RT-PCR

RNA was extracted using MasterPure Yeast RNA Purification Kit (Epicentre). RevertAid H Minus First Strand cNDA Synthesis Kit (Thermoscientific) was used to synthesize cDNA. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to amplify cDNA. Primers used are listed in Table 4. Yeast control primer is U3; control primers for experiments using BeWo cells are 18s and EEF2. For trophoblast RNA extraction, we used a protocol previously described in[3]. In brief, RNA was extracted using Ribozol (VWR International), following manufacturer's protocol. cDNA was created using High Capacity cDNA kit (ThermoFisher Scientific) and Sensifast SYBR Green PCR Master Mix (FroggaBio). cDNA samples were diluted 10X and quantitative PCR was performed using OVOL1, CGB, ERVFD-1 primers (Table 2.4); fluorescence was detected using a CFX96 Connect real-time PCR detection system (Bio-Rad Laboratories). Relative mRNA expression was calculated using the comparative ΔΔCt

method using the comparative mean of reference genes: 18 S Ribosomal RNA (*RNA18SN1)* and Eukaryotic Elongation Factor 2 (*EEF2)* for BeWo cells, and *U3* (for yeast cells). Reference genes were chosen because they have relatively consistent expression levels across cells and experimental conditions.

Table 2.4 List of Primers.

Primers	Sequences
OVOL1/ ZnFC2A/ Δ15OVOL1	F: CCGTGCGTCTCCACGTGCAA R: GGCTGTGGTGGGCAGAAGCC
U3	F: CCCAGAGTGAGAAACCGAAA R:AGGATGGGTCAAGATCATCG
CGB	F: CCTGGCCTTGTCTACCTCTT R:GGCTTTATACCTCGGGGTTG
ERVFD-1	F: CCAAATTCCCTCCTCTCCTC R: CGGGTGTTAGTTTGCTTGGT
TP63	F: CAGATGGACCTGACCAAACC R: AGCTCCGCATCAGCAACTAC
RNA18SN1	F: GCAATTATTCCCCATGAACG R: GGCCTCACTAAACCATCCAA
EEF2	F: AGGCGTAGAACCGACCTTTG R: GACAGCGAGGACAAGGACAA

2.1.8 Yeast Protein Extraction

Cells were incubated overnight in galactose media to induce expression of OVOL1 and then were lysed using the following lysis buffer: 100mM Tris pH 7.5, 200mM NaCl, 1 mM EDTA, 5% glycerol, 1mM Dithiothreitol (DTT).

2.1.9 Mammalian Cell Protein Lysis

Cells were washed with cold phosphate-buffered saline (PBS) and incubated at room temperature with 100 µL of RIPA buffer with protease inhibitor cocktail diluted

1:100 for 10 minutes with shaking. Cells were then scraped off using a cold pipette tip and transferred into a microcentrifuge tube. Cells were lysed using a 27-gauge syringe several times. Samples were pelleted for five minutes at 14 000 rpm. Supernatant was transferred into a new microcentrifuge tube and frozen at -20°C.

2.1.10 Western Blot Analysis

Protein samples were separated using SDS-PAGE (BioRad Mini-PROTEAN TGX Pre-Cast gels, 4-15%) and transferred to nitrocellulose membranes using BioRad Trans-Blot Turbo RTA Transfer Kit. Membranes were blocked using 3% Bovine Serum Albumin (BSA) diluted in PBS containing 0.5% Tween-20 for one hour. The membrane was incubated overnight at 4°C using anti-OVOL1 antibody (Abcam Ab65023, 1:1000 dilution). The following day, the membrane was washed and incubated with AlexaFluor Anti-Rabbit 680 nm. Stain-free gel was imaged to detect total protein (control).

2.1.11 Propidium Iodide Staining

After cells are induced in galactose inducing media, three replicates of 200 ul of each cell culture was added to 1.5 µL tubes. Two additional samples were used as positive and negative controls. Positive control cells were boiled at 100°C for 15 minutes. All samples were resuspended in PBS and PI mixture and incubated at room temperature for 10 minutes. Resuspension solution consisted of 100 µL PBS and 1:200 dilution of propidium iodide (PI) stock solution (1 mg/mL in H_2O). Samples were transferred into a clear bottom 96-well plate and imaged using Gel Doc system (Biorad). The OD_{600nm} was also measured using a plate reader. Percent survival rates were calculated using the ANALYSR program developed by Chadwick et al[4].

2.1.12 Flow Cytometry

Cells were induced overnight in galactose-containing media to induce expression of mCherry Red Fluorescent Protein tagged protein that contained repeats of glutamine (polyQ). A BD Bioscience FACS Celesta flow cytometer with a yellow laser at 561 nm was used to detect red fluorescence. 20,000 cells were analyzed and the median

fluorescence intensities (MFI) was calculated using BD FACS Diva Software. No gates were applied during analysis.

2.1.13 Statistical Analysis

Graphpad Prism (La Jolla CA) was used to calculate all statistical analysis of significance of the Area Under the Curve (AUC) of growth curves was calculated using the two-tailed unpaired t-test or One-way ANOVA followed by Dunnett's Multiple Comparison test. ΔΔCt values were calculated using Ct values generated through quantitative RT-PCR, and two-tailed unpaired t-test was conducted on the 2^ΔΔCt values.

2.2 Results

2.2.1 Expression of *OVOL1* Reduces Yeast Growth

To study OVOL1 function, an OVOL1-yeast model was developed; *OVOL1* was cloned under a *GAL1* galactose-inducible promoter into two CEN plasmids with different auxotrophic markers. One copy (1X-*OVOL1)* or two copies (2X-*OVOL1)* of *OVOL1* were transformed into yeast and growth assays were conducted to determine whether *OVOL1* elicited a change in phenotype (Fig 2.1). A spot assay was performed where yeast was serially diluted and pinned onto agar plates with selective media. When 2X-*OVOL1* was induced, it caused a noticeable decrease in yeast growth, observed by the lower number of yeast colonies (Fig 2.1). We quantified the decrease in growth by measuring growth in liquid (Fig 2.1B). Cells with 2 copies of *OVOL1* showed a significant decrease in growth compared to control (Fig 2.1B, C). Since two copies elicited a change in phenotype, a higher expression model of *OVOL1* was used for the remainder of the project. Western blot analysis was performed to confirm that OVOL1 was being expressed (Fig 2.1D) Indeed only cells expressing 2X-*OVOL1* display a band corresponding to the Ovol1 protein.

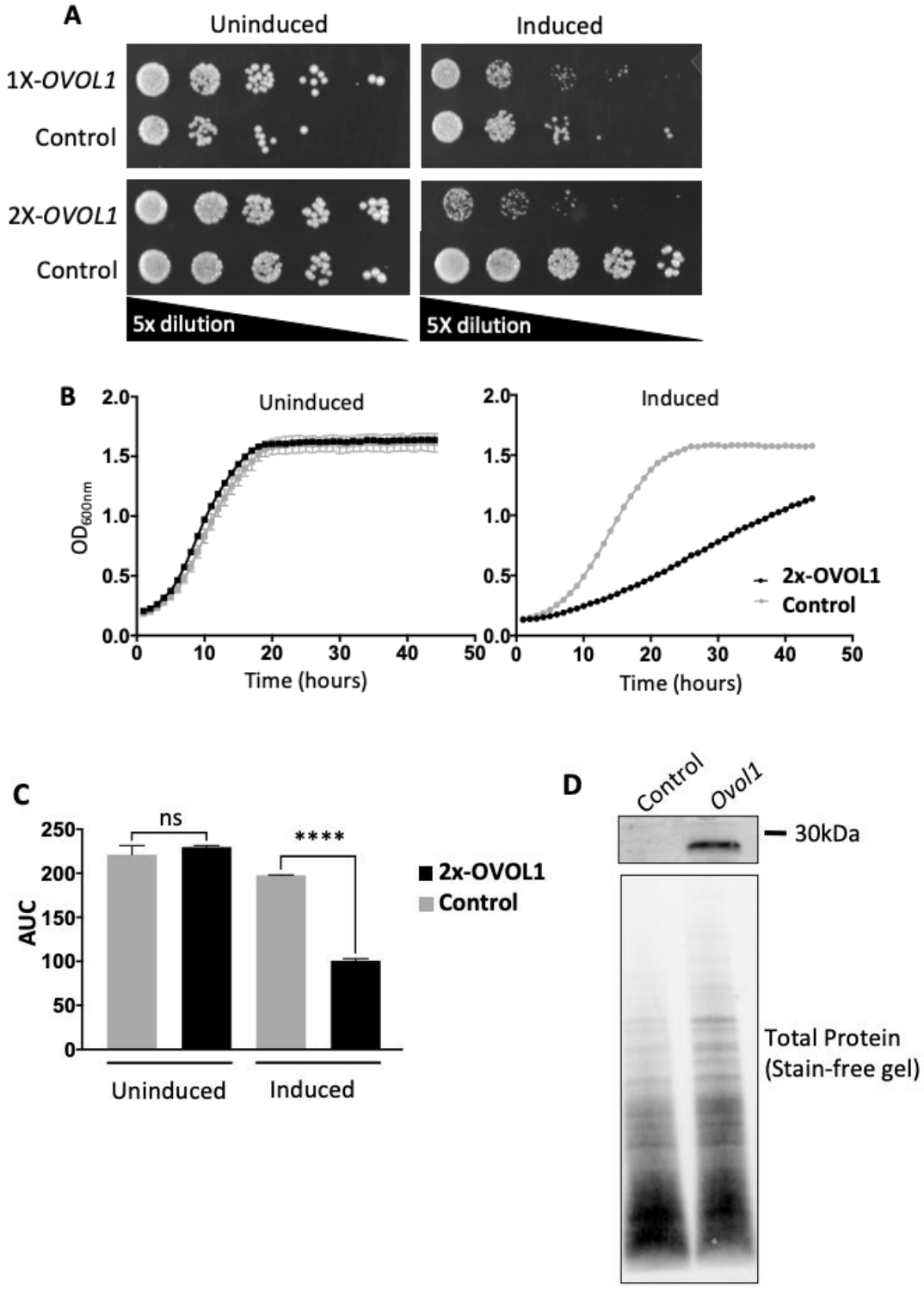
A
Uninduced
Induced
1X-OVOL1
Control
2X-OVOL1
Control
5x dilution
5X dilution
B
Uninduced
Induced
OD600nm
Time (hours)
Time (hours)
2x-OVOL1
Control
C
ns
****
AUC
2x-OVOL1
Control
Uninduced
Induced
D
Control
Ovol1
30kDa
Total Protein
(Stain-free gel)

Figure 2.1 OVOL1 expression reduces yeast growth.

Yeast were transfected with either one copy (1X-*OVOL1)* or two copies (2X-*OVOL1)* of *Ovol1*. Cells were cultured at 30°C in non-inducing media overnight, then were exposed to inducing media the following day for 24 hrs. Cells were then spotted using a 5-times serial dilution onto uninduced and induced agar plates for 48 hrs. Yeast with two copies of *OVOL1* caused a growth defect compared to one copy of *OVOL1*(A). The same cells were diluted, and growth was measured every 15 minutes for 24 hrs in a Bioscreen. Growth curves (B) and area under the curve (AUC) (C) was calculated using GraphPad Prism, and a two-tailed unpaired t–test was performed, non-significant (ns), $p \leq 0.0001$ (****). (D) Western blot confirmed expression of 2X-OVOL1. Mean with standard deviation (SD).

2.2.2 OVOL1 Causes Cell Cycle Arrest in Yeast

Next, we wanted to confirm that the growth defect caused by OVOL1 is due to cell death. A propidium iodide stain was performed to determine whether expression of OVOL1 results in cell death. Cells expressing OVOL1 showed a significant but modest decrease in percent survival (15% difference) compared to control (Fig 2.2A). Cells expressing ZnFC2A did not show a difference in percent survival compared to control (Fig 2.2). This further confirms that OVOL1 requires a functional zinc finger domain to inhibit growth in yeast. Furthermore, there is no difference in the amount of fluorescence between PI-stained cells expressing OVOL1, ZnFC2A and control plasmid (Fig 2.2B). To determine whether these cells are permanently growth arrested or nonviable, we performed a regrowth assay. After cells have been induced overnight in galactose media (where OVOL1 is induced), they are spotted onto YPD plates (no *OVOL1* induction). There is no change in the growth of control, OVOL1 and ZnFC2A cells (Fig 2.2C). Overall, this suggests that the growth defect caused by OVOL1 is due to transient inhibition of cell growth.

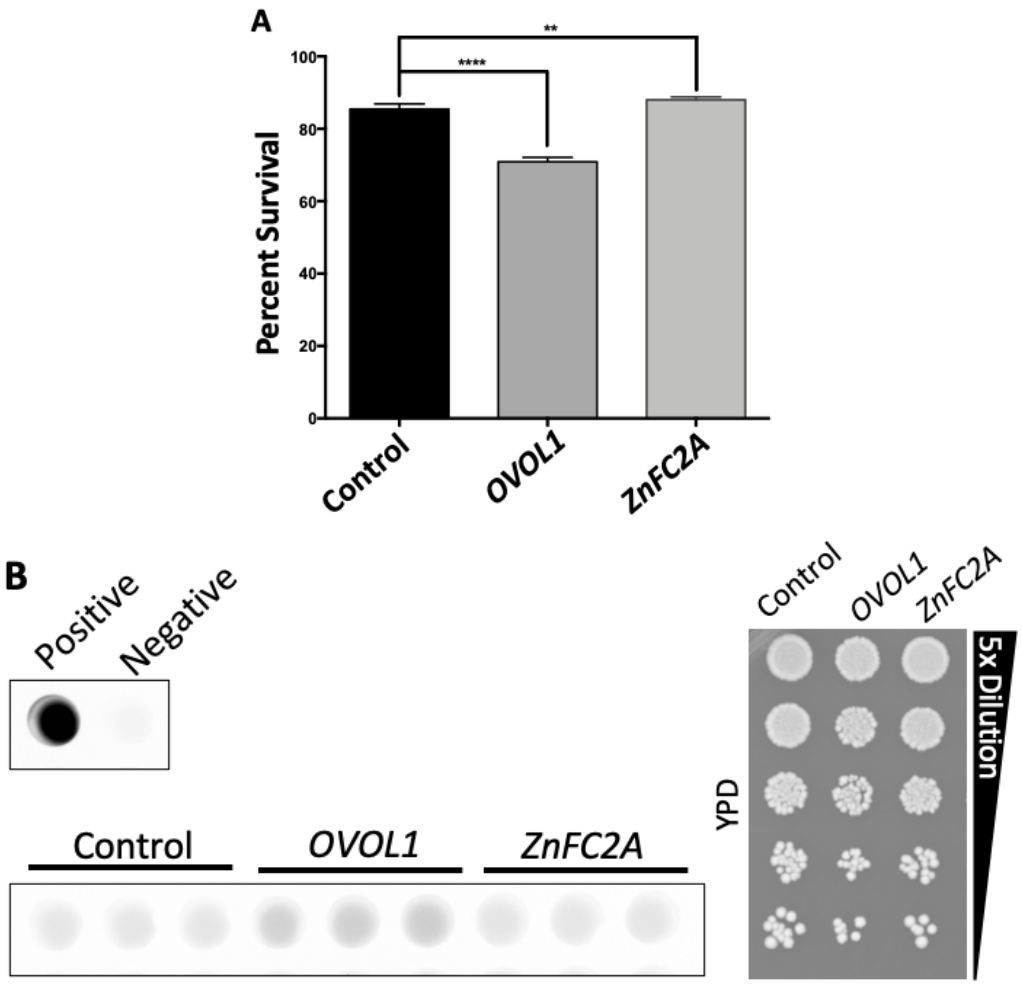

Figure 2.2 OVOL1 expression does not cause cell death.

Cells expressing wild-type *Ovol1* (*OVOL1*) or zinc finger mutated *Ovol1 (ZnFC2A*) were grown in induction media overnight, cells were stained with propidium iodide and fluorescence was imaged using a Gel Doc system. Percent survival was calculated suing protocol from Chadwick et al (2016). Positive control (positive) are cells that have been boiled for five minutes, and the negative control (negative) are cells that have not been stained with PI. One-way ANOVA followed by Dunnett's Multiple Comparison test was performed on GraphPad Prism. *OVOL1* caused a significantly lower percent survival than control, $p \leq 0.0001$(***), and *ZnFC2A* cells, $p \leq 0.01$(**). Mean with SD.

2.2.3 OVOL1 Activity is Dependent on Binding to DNA and HDACs

Yeast cells expressing OVOL1 with the zinc finger mutations (ZnFC2A) showed no difference in phenotype compared to control when cells are induced in galactose media (Fig. 2.3A). This confirms that the growth defect is due to OVOL1 having functional zinc-finger binding activity. OVOL1 also contains an N-terminal 9-amino acid SNAG domain, which has been implicated in recruiting HDAC1[5]. To determine if the SNAG domain is required for OVOL1 to suppress yeast growth, we cloned the Δ15-OVOL1 plasmid into yeast, and assayed OVOL1's effect on cell growth. The SNAG-deletion in OVOL1 did not affect growth in wild-type yeast cells (Fig 2.3 A, B). Taken together, these results suggest that the inhibition of cell growth in yeast is dependent on zinc finger domain-mediated DNA binding of OVOL1, as well as the SNAG domain. The requirement of a SNAG domain may indicate that OVOL1 recruits HDACs to genes implicated in cell growth, thereby deacetylating and repressing these genes.

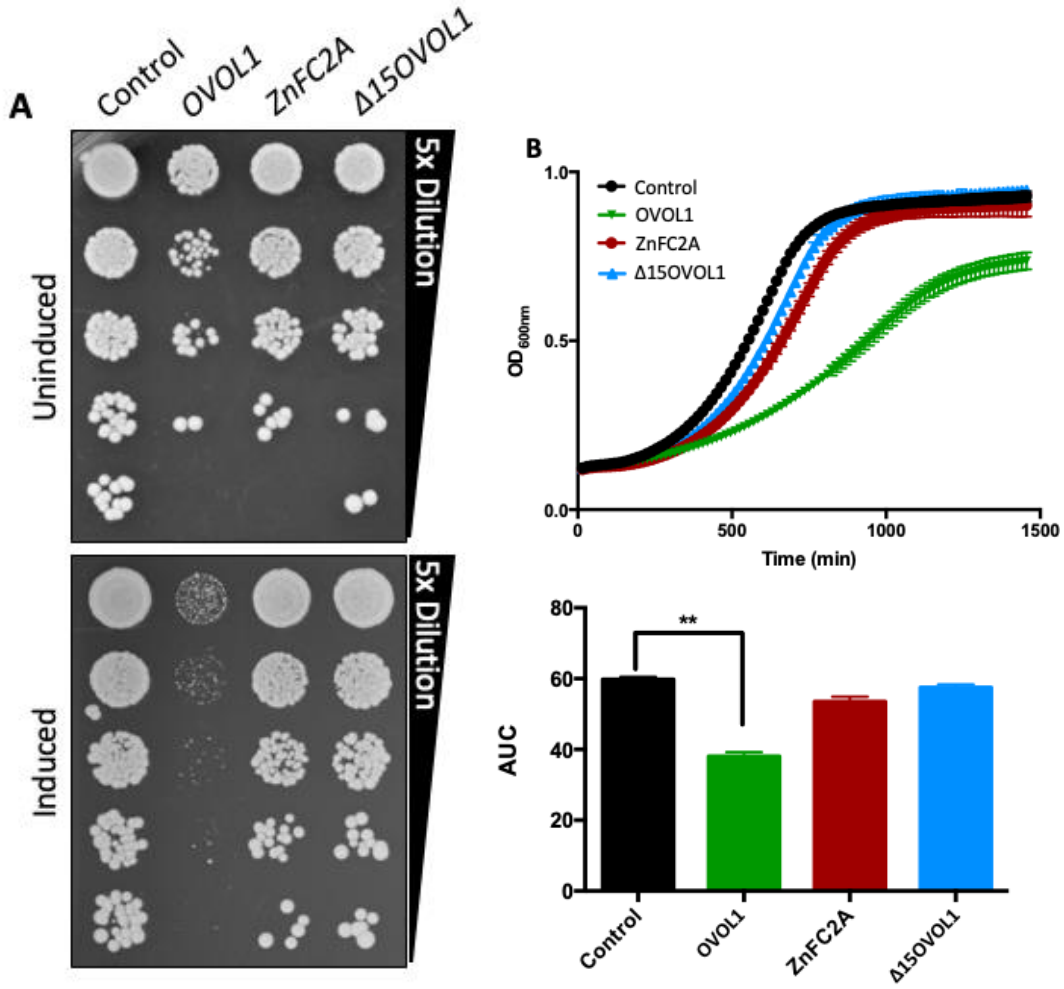

Figure 2.3 Mutations in OVOL1 have no effect on yeast growth.

Cells expressing *Ovol1* (OVOL1), zinc finger-mutated *Ovol1 (*ZnFC2A), and SNAG-deleted *Ovol1* (Δ15OVOL1*)* were serially diluted and spotted on glucose (uninduced) and galactose (induced) plates. *Ovol1* mutations did not elicit a growth defect (A). Liquid growth assay was also performed, and growth curves and area under the curve (AUC) were generated by GraphPad Prism. Growth was significantly decreased in OVOL1 expressing cells, $p \leq 0.01$ (**) (B). Mean with standard SD. One-way ANOVA followed by Dunnett's Multiple Comparison test using GraphPad Prism.

2.2.4 Class II HDACs Play a Role in OVOL1 Regulation of Cell Proliferation

Since HDACs are required for BeWo trophoblast differentiation[3], we wanted to investigate whether OVOL1 requires the presence of HDACs in yeast to regulate cell proliferation. HDAC deletion strains were used to determine whether there is a genetic interaction between OVOL1 and HDACs. Initially, three important HDAC proteins from class I (*rpd3Δ*), II (*hda1Δ*) and III (*sir2Δ*) were used. Growth assays were performed to determine whether there is a change in growth phenotype. When *OVOL1* was induced by placing cells in galactose media, there was a growth defect evident in control, *rpd3Δ* and *sir2Δ* cells, but no growth defect in *hda1Δ* (Fig 2.4 A). Furthermore, the other class II HDAC proteins, *hda2* and *hda3*, also showed a significant rescue in growth compared to control (Fig 2.5 A).

To further determine whether other HDACs belonging to the three HDAC families were implicated in regulating cell growth by OVOL1, we transformed OVOL1 constructs into the remaining HDAC deletion strains, and growth assays were performed (Fig 2.5 and 2.6). Both class I and III showed significant decrease in cellular growth when OVOL1 was induced, but the growth defect was less pronounced in yeast lacking class II HDACs (Fig 2.5 and 2.6). Collectively, these results indicate that OVOL1 interacts with class II HDACs to reduce cell growth.

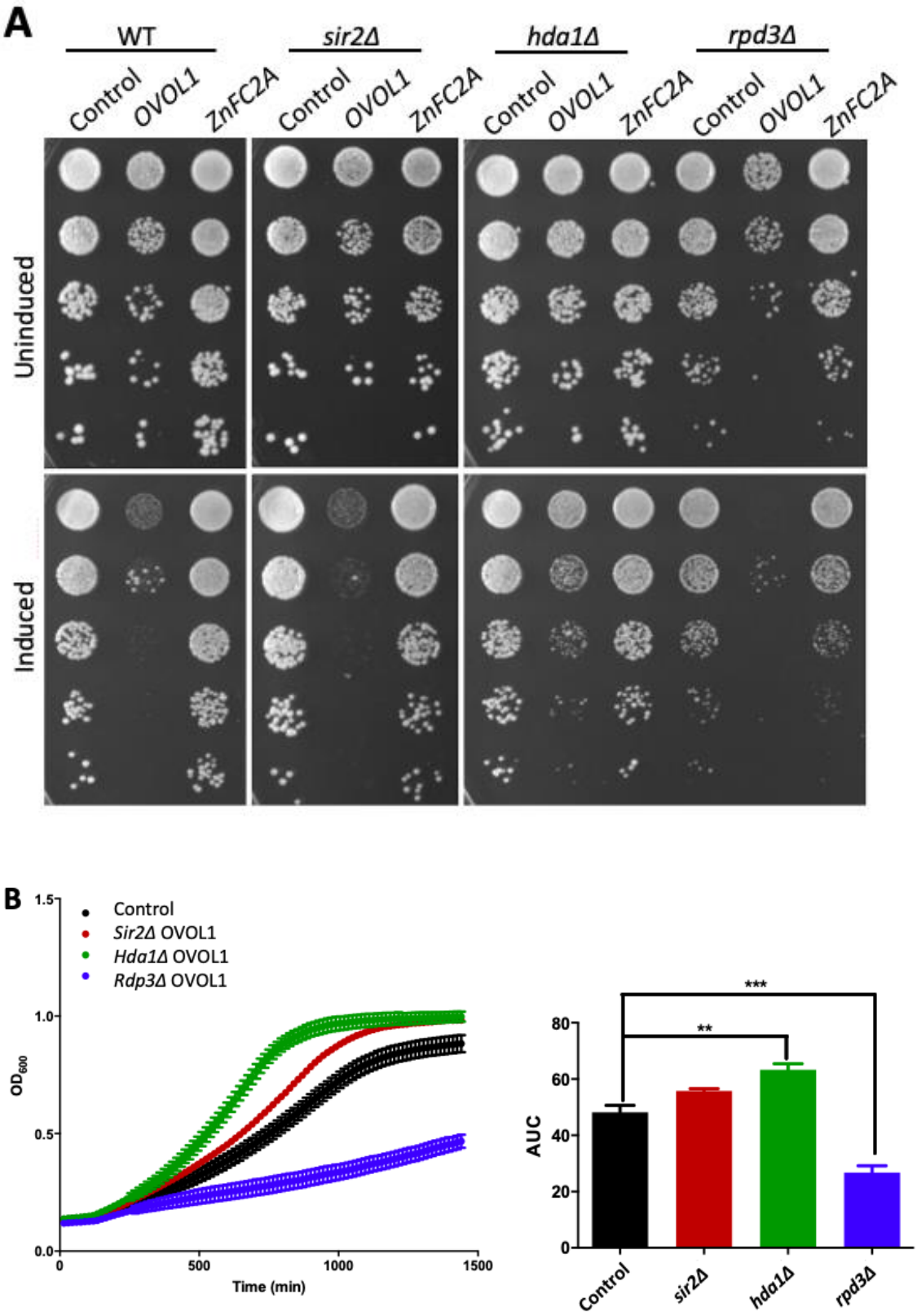
A
WT
sir2Δ
hda1Δ
rpd3Δ
Control
OVOL1
ZnFC2A
Uninduced
Induced
B
Control
Sir2Δ OVOL1
Hda1Δ OVOL1
Rdp3Δ OVOL1
OD600
Time (min)
AUC
***
**
Control
sir2Δ
hda1Δ
rpd3Δ

Figure 2.4 Deletion of HDA1, a class II HDAC, specifically rescues the growth defect caused by OVOL1.

Control, *Ovol1*, and *ZnFC2A* expressing plasmids were transformed into wild-type (WT), class I (*sir2Δ*), class II (*hda1Δ*), and class III (*rpd3Δ*) HDAC deletion strains. Cell growth was assessed by serial dilutions onto glucose and galactose plates. *hda1Δ* expressing OVOL1 expressing cells rescued the growth defect seen in the other strains expressing OVOL1 (A). Liquid growth assays were further used to confirm growth rescue, there was a significant difference in growth between *hda1Δ* expressing OVOL1 and control cells expressing a plasmid deficient of *OVOL1*, $p \leq 0.01$ (**) (B). One-way ANOVA followed by Dunnett's Multiple Comparison Test.

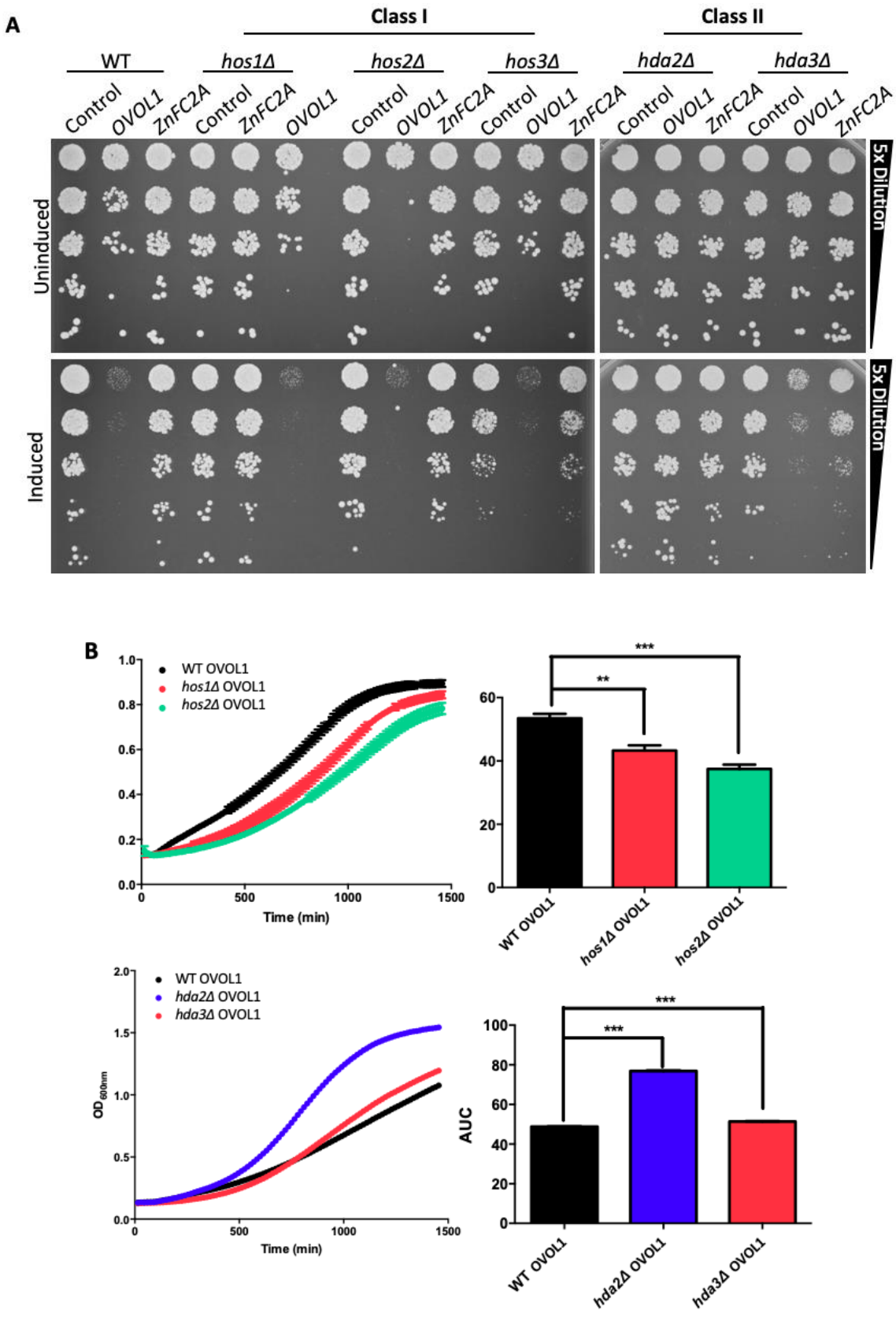
A
Class I
Class II
WT
hos1Δ
hos2Δ
hos3Δ
hda2Δ
hda3Δ
Control
OVOL1
ZnFC2A
Uninduced
Induced
5x Dilution
B
WT OVOL1
hos1Δ OVOL1
hos2Δ OVOL1
Time (min)
hda2Δ OVOL1
hda3Δ OVOL1
OD600nm
AUC
***
**

Figure 2.5 Deletions of class II HDACs (*hda2, hda3*) alleviates growth defect caused by OVOL1.

(A) Spot assay showing wild type strain, class II HDACs (*hda2Δ* and *hda3Δ*), and class I HDACs (*hos1Δ* and *hos2Δ)* deletion strains expressing either: control, OVOL1, or ZnFC2A plasmid. (B) Area under the curve (AUC) was determined by analyzing the growth curves using GraphPad Prism. *Hos1Δ* and *hos2Δ* expressing OVOL1 cells grew significantly less than wild type (WT) strain, $p \leq 0.01$(**) and $p \leq 0.001$(***), respectively. Class II HDACs, both *hda2Δ* and *hda3Δ* cells grew significantly better than WT OVOL1 cells, $p \leq 0.001$(***). One-way ANOVA followed by Dunnett's Multiple Comparison test. Mean with SD.

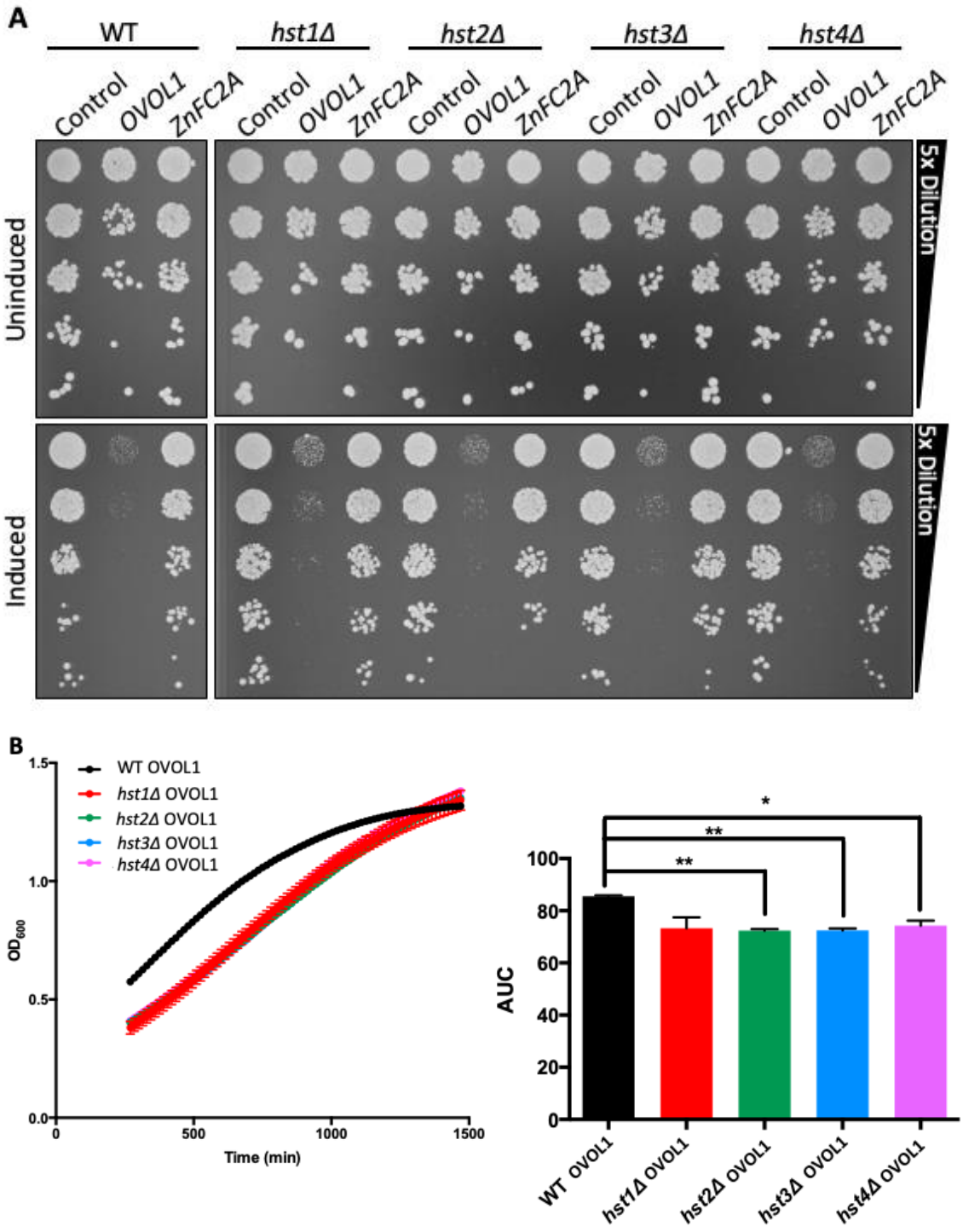
A
WT
hst1Δ
hst2Δ
hst3Δ
hst4Δ
Control
OVOL1
ZnFC2A
Uninduced
Induced
5x Dilution
B
WT OVOL1
hst1Δ OVOL1
hst2Δ OVOL1
hst3Δ OVOL1
hst4Δ OVOL1
OD600
Time (min)
AUC
*
**
**
WT OVOL1
hst1Δ OVOL1
hst2Δ OVOL1
hst3Δ OVOL1
hst4Δ OVOL1

Figure 2.6 Yeast carrying class III HDAC deletions display reduced growth when OVOL1 is expressed.

(A) Growth assays were performed using wild type and class III HDAC deletions (*hst1Δ hst2Δ, hst3Δ,* and *hst4Δ*) expressing control, OVOL1, or ZnFC2A plasmids. (B) Using the growth curves generated using a BioScreen Plate Reader, area under the curve (AUC) was calculated using GraphPad prism, one-way ANOVA followed by Dunnett's Multiple Comparison test. Cell growth of *hst2Δ, hst3Δ,* and *hst4Δ* is significantly affected, $p \leq 0.01$ (**), $p \leq 0.05$ (*). Mean with SD.

2.2.5 OVOL1 is Expressed in HDA1-Deletion strain

To ensure that our results are not due to reduced OVOL1 expression in yeast strains lacking class II HDACs, we confirmed that *OVOL1* was expressed at the RNA and protein levels. qRT-PCR was conducted to reveal that *OVOL1* is expressed in control and to even higher extent in *hda1Δ* cells (Fig 2.7A). Next, a western blot analysis revealed that OVOL1 protein is present in both the control and *hda1Δ* strains; however, reduced protein expression of OVOL1 was evident when the zinc finger domains were mutated (ZnFC2A) (Fig 2.7 B). *OVOL1* was also tagged to a green fluorescent protein (GFP) reporter to determine cellular localization. We detected OVOL1-ymsfGFP both in the nucleus and cytoplasm (Fig 2.7 C).

Since *hda1Δ* strains show a more robust expression of *OVOL1*, we wanted to test the *GAL1* promoter activity to determine whether it is different between the deletion and wild-type strain (Fig 2.8). An mCherry-fluorescent reporter under the control of the *GAL1* promoter was transformed into wild-type and *hda1Δ* strains. Flow cytometry was performed to determine the level of fluorescence when cells were induced in galactose media. No change in fluorescence intensity was detected between wild-type and *hda1Δ* cells, therefore, the change in *OVOL1* mRNA and protein levels cannot be attributed to different *GAL1* driven transcriptional activity (Fig 2.8).

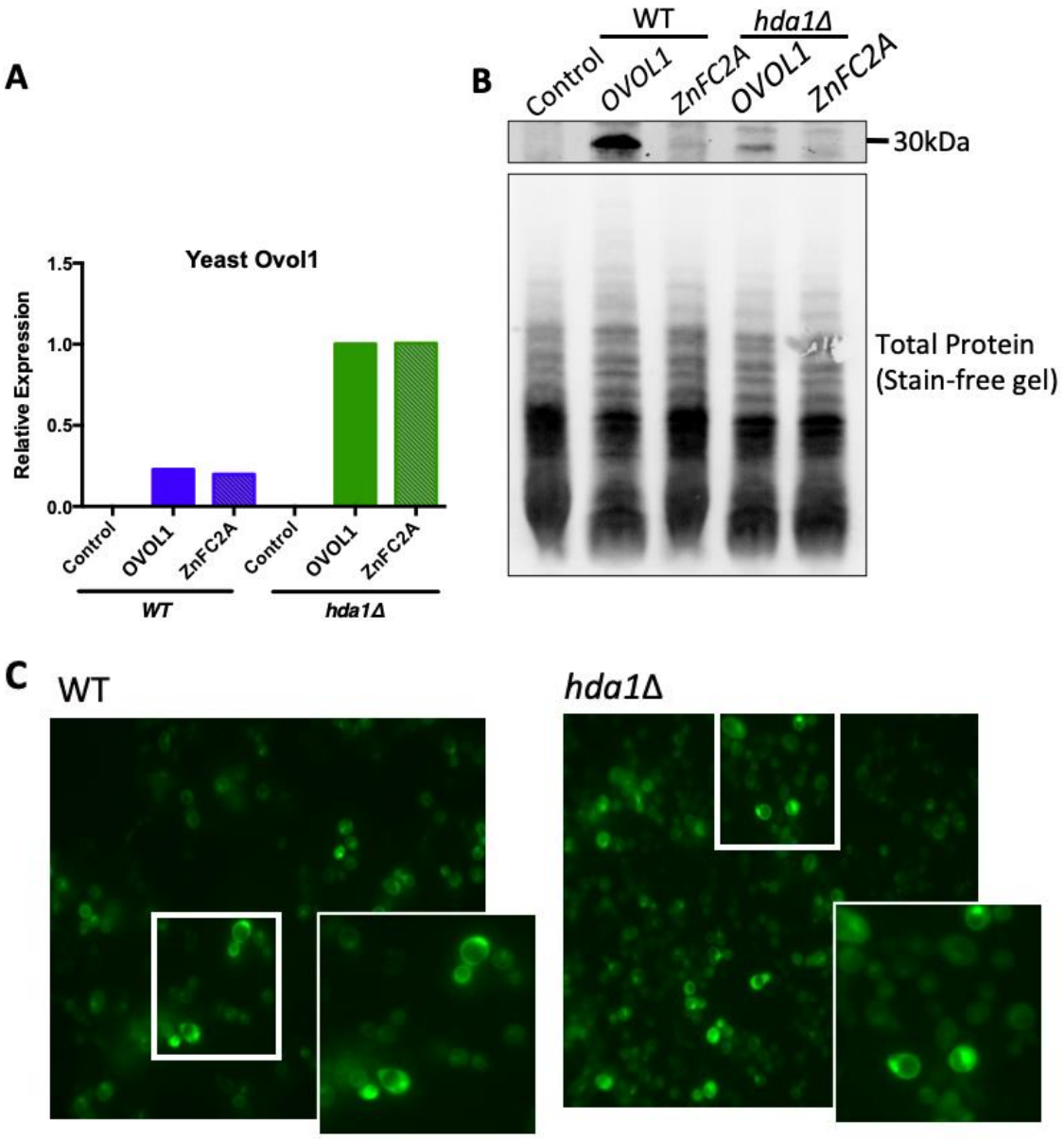

Figure 2.7 OVOL1 is expressed at both the RNA and protein level in wild type and *hda1Δ* cells.

(A) qRT-PCR was conducted to assess the expression of *OVOL1*. Western blot analysis (B) was performed to confirm protein expression of OVOL1. (C) Representative fluorescent microscopy images of control and *hda1Δ* cells expressing *Ovol1*-GFP. Please note the strong nuclear staining in OVOL1-GFP cells.

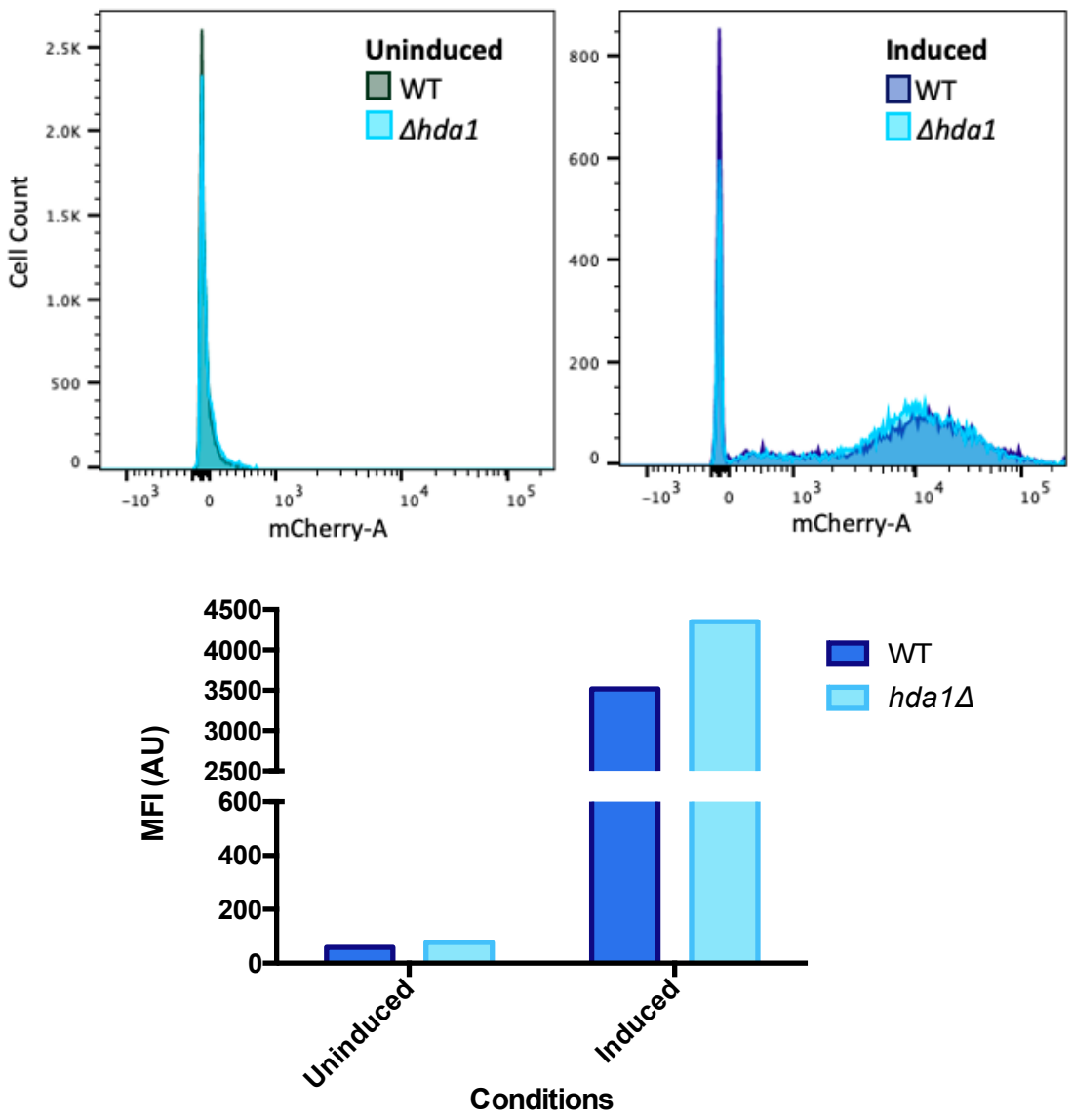

Figure 2.8 The GAL1 inducible promoter activity is similar between wild type and *hda1Δ* cells.

(A) Wild type and *hda1Δ* cells expressing an mCherry fluorescence reporter were analyzed by flow cytometry. (B) Median fluorescent intensity (MFI) of the cells were calculated from fluorescent data (geometric mean) acquired using flow cytometry. There was no statistically significant difference in *GAL1*-promoter activity between wild type and *hda1Δ* cells.

2.2.6 Ectopically Expressing OVOL1 in Human BeWo trophoblasts

Before determining how overexpressing OVOL1 will affect BeWo trophoblast proliferation, we wanted to ensure BeWo cells are able to upregulate differentiation markers in the presence of cAMP. *OVOL1* and *ERVFRD1* are trending towards an increase in expression, and *CGB* shows a significant increase in expression in cells treated with cAMP compared to control cells (Fig 2.9). However, there was no significant change in expression of TP63, which is a stem cell associated gene (Fig 2.9). BeWo cells maybe be undergoing differentiation under induction media; however, more replicates are required. We then used BeWo cells to determine the effect of ectopically overexpressing *OVOL1* on trophoblast differentiation. To develop such a model, I cloned *OVOL1* into a pEF-GFP vector. qRT-PCR was performed to detect increased expression of *OVOL1* mRNA levels in cells transfected with the pEF-*OVOL1* vector (Fig 2.10). BeWo cells exhibited a significant increase in *OVOL1* expression compared to control cells, along with an increase in expression of a differentiation-associated transcript, *ERVFRD-1* (Fig 2.10). Lastly, expression of *CGB*, a hormone produced by syncytiotrophoblast, increased when OVOL1 protein is overexpressed compared to control cells (Fig 2.10). Thus, ectopic expression of *OVOL1* maybe sufficient to drive cells to undergo at least partial differentiation by increasing expression of syncytial-associated genes.

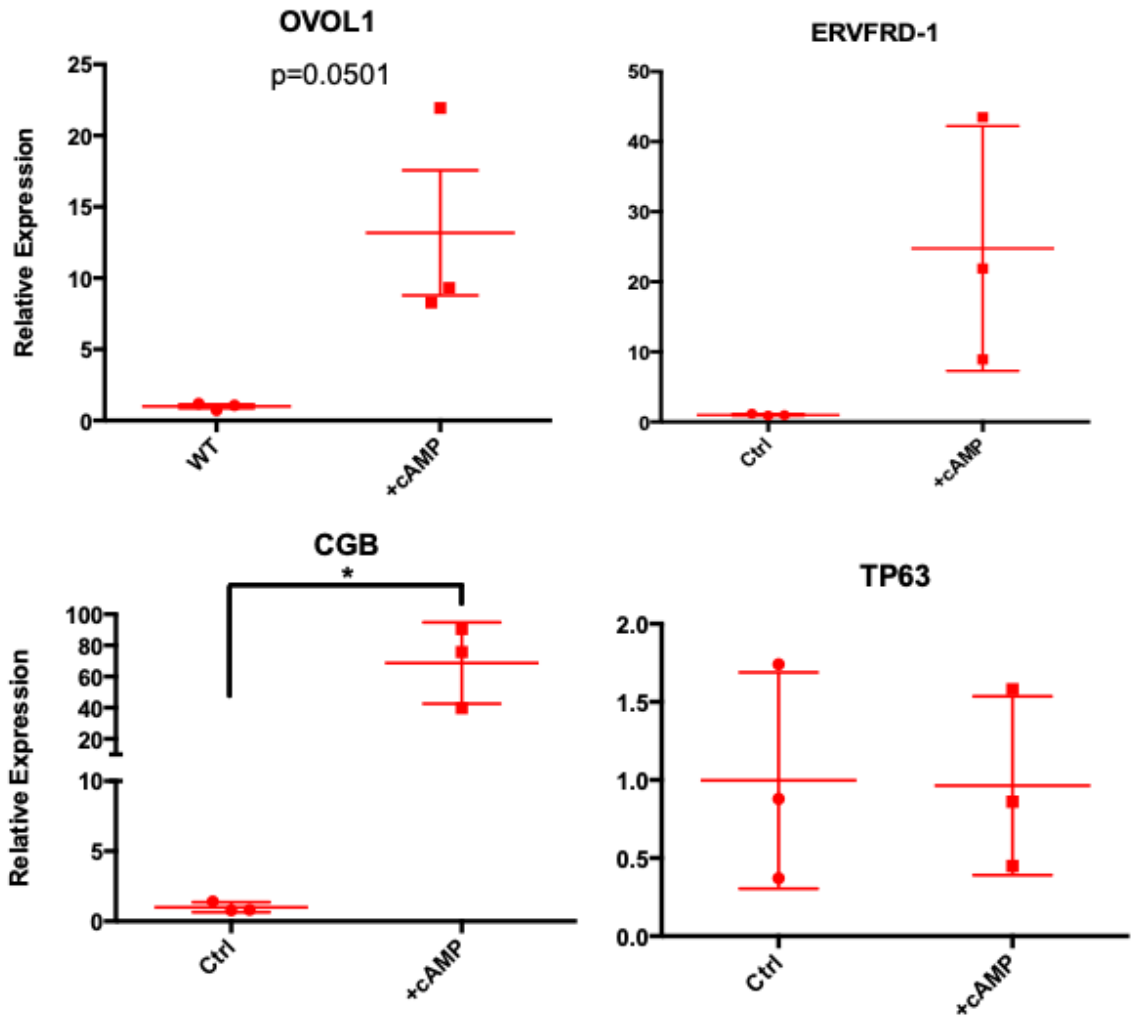

Figure 2.9 Treatment of BeWo Trophoblast with cAMP upregulates genes associated with differentiation.

BeWo cells were either untreated (Ctrl) or treated with 10 µL/ng of cAMP (+cAMP) for 48 hrs before cells were lysed and RNA was isolated. qRT-PCR was conducted to determine expression levels of *OVOL1*, the cytotrophoblast marker *TP63*, and the syncytiotrophoblast-associated genes *CGB* and *ERVFRD-1*. Expression of the following markers was significantly increased in cAMP treated cells: *OVOL1* ($p \leq 0.01$,**), ERVFRD-1 ($p \leq 0.001$,***) and CGB ($p \leq 0.0001$,****). Unpaired t-test was conducted using Graphpad Prism. Mean with SD. N= 3.

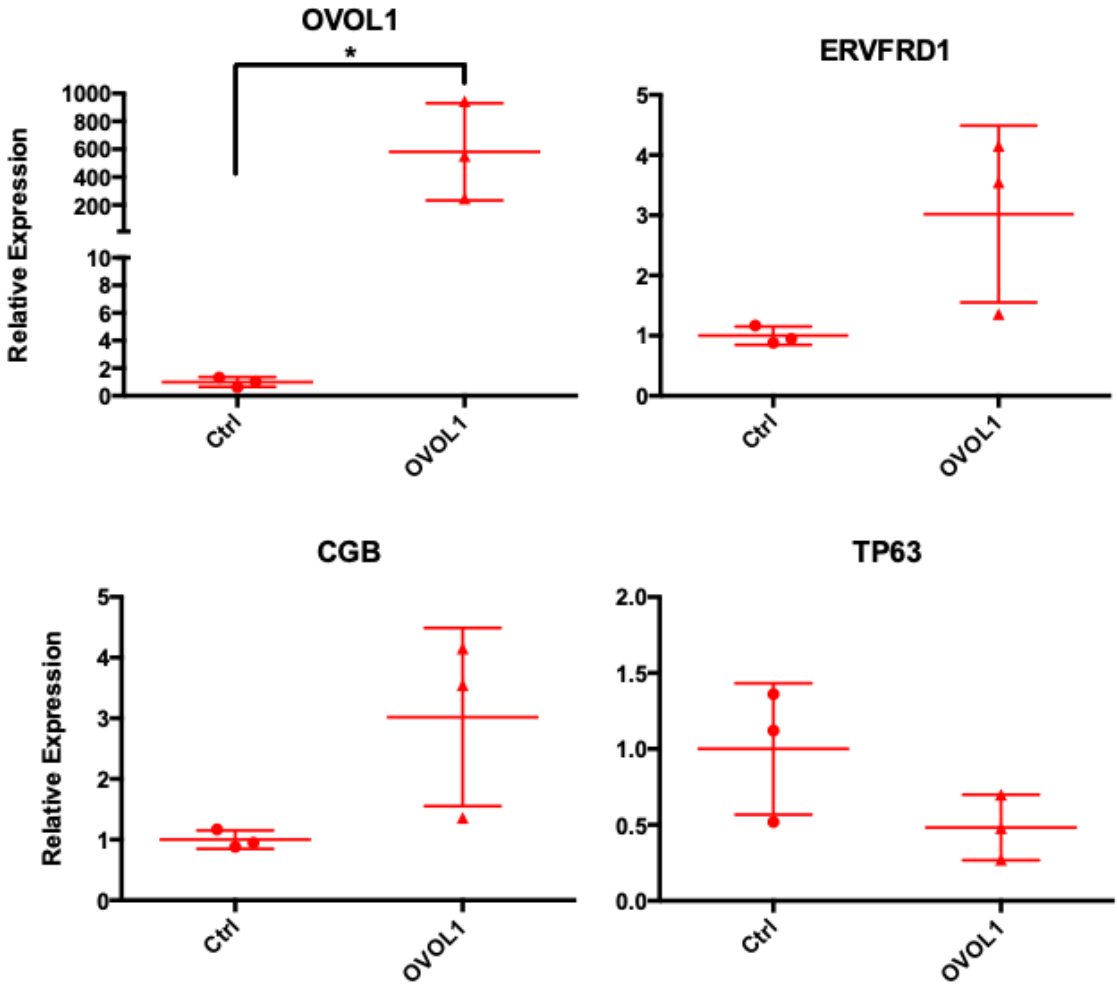

Figure 2.10 Ectopic expression of OVOL1 partially drives expression of genes associated with differentiation in BeWo cells.

BeWo cells were transfected with pEF-GFP plasmid (Ctrl) or pEF-*OVOL1* (Ovol1). Cells were lysed for RNA and qRT-PCR was used to detect expression of *Ovol1*, *ERVFRD-1*, *CGB* and *TP63*. Using GraphPad Prism, significance was determined using an unpaired t-test. Cells overexpressing Ovol1 has significantly more *OVOL1* and *ERVFRD-1* expression under 10 µL/ng of cAMP ($p \leq 0.05$), There was no significance expression of *CGB* and *TP63* between ctrl and *OVOL1* overexpressing cells. Mean with SD. N= 3.

2.1 Discussion

The world faced a pandemic that affected research across the world. Due to university closures, my work in BeWo cells had to come to a halt unexpectedly. I will list the remaining experiments that were needed to complete my thesis. To confirm that overexpression of OVOL1 is sufficient to cause cell fusion and increase of expression of syncytial-associated genes first, a few more replicates are needed to ensure that ectopically overexpressing OVOL1 in BeWo trophoblasts is correlated with increased gene expression of *ERVFRD-1* and *CGB*. Immunofluorescence experiments would be conducted on BeWo trophoblasts with OVOL1 overexpression to look at percent fusion. Fluorescent-labeled antibodies targeting hCGB and CDH1 (or Actin) will be used to look at cellular fusion, and cells will be counted to determine percent fusion occurring compared to cells transfected with a control plasmid.

Second, to determine whether HDAC function is required for OVOL1 to induce differentiation. OVOL1 will be overexpressed, and one HDAC protein from class II family will be targeted at a time using siRNA. Western blot analysis will be used to confirm downregulation of class II HDACs, qRT-PCR to determine whether *ERVFRD-1* and *CGB* are upregulated under OVOL1 overexpression in the absence of class II HDACs, and immunofluorescent to observe cell fusion.

Lastly, co-immunoprecipitation assay would be conducted to determine whether class II HDAC protein directly interacts with OVOL1, and RNA-seq would be used to determine expression of genes that are altered during overexpression of OVOL1.

My work is the first to use yeast *Saccharomyces cerevisiae* to investigate how OVOL1 affects cell proliferation. Yeast have been widely used to model human genes and assess protein functionality and their effect on cell growth [6]. The absence of an OVOL1 homolog makes yeast an attractive model to study the phenotype associated with OVOL1expression on growth and proliferation. Thus, we used yeast to study the effect of OVOL1 on cell growth. When trophoblasts differentiate, they increase expression of OVOL1 which redirects cells from a proliferating to a differentiating state, which is associated with activation of genes that promote differentiation (*ERVFDR-1* and *CGB*) and repression of proliferation-associated genes (*TP63*) [7]. It was interesting that ectopic expression of OVOL1 in yeast caused reduced cell growth and provides further evidence

that the primary function of OVOL1 in differentiating cells is the repression of proliferation. OVOL1 has been characterized to transcriptionally represses genes through two processes; indirectly repressing gene expression by recruiting co-repressors or by directly binding to promoters upstream of target genes [5,8]. OVOL1 was able to repress the *OVOL1* promoter by binding to a specific target sequence, CCGTTA, and recruit HDACs to *OVOL1* promoter to cause gene repression [5]. How OVOL1 interacts with histone deacetylase complexes, and whether this interaction is important for the regulation of cell growth, has yet to be determined. We wanted to investigate HDAC-OVOL1 relationship with regards to cellular growth. Therefore, we predicted that histone deacetylase complexes may be acting with OVOL1 to regulate cellular proliferation and growth in yeast.

Since OVOL1 causes a growth defect in yeast, we determined whether the absence of HDACs can rescue growth. Using some HDAC deletion strains from a deletion library, we cloned the OVOL1 gene into class I, II and III HDAC deletion strains to determine whether there is an underlying genetic interaction between HDACs and OVOL1. We found that deletion of class II HDACs alleviated the growth defect induced by OVOL1. In addition, there was a difference in the degree of rescue between the three HDACs within class II. *Hda2Δ* and *hda1Δ* significantly mitigated the growth defect caused by OVOL1 expression compared to *hda3Δ.* The difference in level of growth may be due to a number of reasons.

First, class II HDACs work together by forming a tetrameric complex to deacetylate histones and share similar genetic interactions[9,10]. Therefore, if one of the HDAC proteins is absent from the complex, it could render the complex unable to optimally remove acetyl groups from histones. Second, each subunit within the class II HDAC complex has unique genetic interactions and function in different cellular pathways; only 7% of interactions are shared amongst the three proteins when a genome wide screen was conducted in yeast [11]. For example, HDA2 plays a bigger role in peroxisome function and maintenance than the other two class II HDACs, it also interacts with the HDAC complex by directly binding to HDA3 [12]. HDA3 is important in nuclear/cytoplasmic transport, and directly interacts with HDA1 [11,12]. HDA1 interaction with HDA2-HDA3 subunit is necessary because HDA2 has a prosthetic group involved in transferring an acetyl group

during histone deacetylation, and HDA3 is important in bringing the subcomplex HDA2-HDA3 with HDA1 homodimer [12,13]. Both HDA2-HDA3 are essential because they are important in histone recognition and altering HDA1 stoichiometry thereby activate the catalytic subunit found in the HDA1 protein [12]. Similar to yeast class II protein interaction, human class II HDACs show analogous interactions within a multiprotein complex [14].

Overall, our data suggest that class II HDACs are necessary for OVOL1 to inhibit cellular proliferation. Interestingly, class II HDACs seem to play a role in regulating activity of pivotal transcription factors during trophoblastic fusion [15]. Inhibiting HDACs in BeWo trophoblasts prevented their fusion and differentiation into syncytiotrophoblast [3]. Therefore, HDACs may be necessary in regulating placental development and the integrity of the placental exchange surface.

When *HDA1* is deleted in yeast, OVOL1 protein levels decrease compared to wild-type cells; OVOL1 protein level is further reduced when the DNA binding region is mutated. Since *OVOL1* expression levels remained unchanged between OVOL1 and ZnFC2A, our data suggests that in order for OVOL1 to function, it needs to be stabilized by binding to HDA1. Thus, in the absence of HDA1 and when the zinc finger domain is mutated there is a decrease in OVOL1 and ZnFC2A protein levels. Protein stabilization by HDACs is similarly observed with other transcription factors such as HIF1α and with class III HDACs like SIRT1. SIRT1 directly binds to HIF1α to deacetylate and stabilize it during hypoxia, which mediates increase in genes promoting cancer cell invasion [16,17].

Histone deacetylase complexes can also deacetylate non-histone proteins such as transcription factors, which can act to stabilize or destabilize them. Human HDAC 1, 3, 4 and 5 (HDAC 4 and 5 are class II HDACs) are expressed throughout the placenta, and during trophoblast cell fusion, they act to deacetylate glial cell missing homolog 1 (GCM-1) to destabilize it and inactivate it[15]. Class II HDACs have been shown to be essential in regulating trophoblast differentiation, invasion and migration. Chang et al. found that when cytotrophoblast cells are induced to differentiation, syncytiotrophoblast cells had increased expression levels of HDAC5 and GCM-1, and both had increased colocalization in the nucleus. HDAC5 can also be phosphorylated, which causes it to be exported outside of the nucleus and thereby prevented from altering gene expression and

preventing excessive fusion[18]. Similarly, HDAC9 is localized in the nucleus of syncytiotrophoblast and is critical for trophoblast invasion and migration during early stages of blastocyst implantation[19]. It does so by repressing expression of a matrix metalloproteinase inhibitor (TIMP3), thereby allowing matrix metalloproteinases (MMPs) to degrade and remodel the decidua to facilitate for further blastocyst invasion[19]. Interestingly, HDAC5 is downregulated in preeclamptic placentas[19]. Lastly, HDAC5 is important in regulating EMT switch in trophoblast cells during implantation[20]. In addition, I have shown that class II HDACs function and genetic interaction with OVOL1 is important for cellular proliferation.

We found that *ERVFRD-1* expression increased when OVOL1 was overexpressed in BeWo cells. Since inhibiting HDACs reduces BeWo cell differentiation[3], it is possible that HDACs may be regulating OVOL1 protein levels through post translational modification by deacetylating OVOL1 and rendering it active. Therefore, OVOL1 may be unstable when the zinc finger domain is mutated or when it is being acetylated (in the absence of HDA1).

Elevated OVOL1 levels in trophoblast cells is associated with terminal differentiation into syncytiotrophoblast and its mechanism of action is not well explored. In order for trophoblasts to differentiate they require transcription factors to turn off genes involved in proliferation. Our yeast findings indicate a genetic interaction between class II HDACs and OVOL1. We believe that OVOL1-HDAC regulation of cellular growth and proliferation in yeast is similar in BeWo trophoblasts.

2.2 Conclusion

The next steps to determine overexpression of *OVOL1* in BeWo trophoblast cells was to conduct experiments that determined whether overexpression of *OVOL1* was sufficient to induce differentiation in trophoblast cells in the absence of specific HDAC proteins. To determine whether HDACs are required for differentiation, we can use TSA to block HDAC activity or siRNA to knockdown specific HDACs in conjunction with overexpressing *OVOL1*. CRISPR Screen can be conducted to identify genes which can help define the role of OVOL1 and the cellular pathway it acts through. RNA Sequencing (RNA-Seq) can also be used to determine change in transcript levels of genes involved in differentiation under *OVOL1* overexpression.

Nair et al.[5] and Renaud et al.[7] both found that OVOL1 represses gene expression by binding to a conserved sequence, CCGTTA, within the promoter regions of genes like MYC and ID2. We can perform a chromatin immunoprecipitation (ChIP) assay on BeWo trophoblasts that have ectopic overexpression of *OVOL1* in the absence of HDACs. This will allow us to answer questions like: Will OVOL1 still bind to CCGTTA of genes involved in cell proliferation in the absence of HDACs? Can OVOL1 directly or indirectly (through HDACs or HATs) regulate expression of differentiation-associated genes like *ERVFRD-1*? When the DNA binding region is mutated, OVOL1 was unable to rescue the growth defect seen in yeast. We can use a similar experiment in BeWo cells to determine whether the zinc finger region is necessary when *OVOL1* is overexpressed.

We believe that cellular proliferation and differentiation is regulated by OVOL1 and HDAC proteins interacting to regulate gene expression. In order to determine whether OVOL1 exclusively binds to class II HDACs, we perform a immunoprecipitation (IP) assay to determine whether OVOL1 binds specific class II HDACs or to other classes of HDACs in BeWo trophoblasts to cause gene repression. Protein-protein interaction can also be examined using Fluorescence Resonance Energy Transfer (FRET) or Split-Ubiquitin Yeast Two Hybrid (Split-Ubi) assay.

HDACs modify acetylation on histone proteins, to determine which acetylation sites are affected, mass spectrometry is a great tool to determine the sites of acetylation and whether acetylation levels change when *OVOL1* is overexpressed independent of HDAC inhibition or with class II HDAC inhibition. HDACs also regulate activity of

non-histone proteins, and by performing mass spectrometry we can determine whether lysine sites on OVOL1 and other proteins are deacetylated during BeWo trophoblast differentiation.

Future work will investigate whether OVOL1 is directly recruiting class II HDACs to promoter regions of DNA involved in cellular growth and proliferation. If OVOL1 proteins are not stabilized by HDAC binding, the proteins could be quickly degraded and cell growth will not be affected . Since protein degradation is highly conserved across eukaryotes, including in yeast. We can inhibit proteasomes and HDAC activity in yeast and BeWo cells to determine the protein turnover rate of OVOL1, and whether that affects both cellular proliferation and differentiation. We can perform a cycloheximide (CHX)-chase assay in BeWo cells. Cells will be collected after addition of CHX and cAMP after certain time points for 48 hrs to determine the level of OVOL1 present in cells that are differentiating. We can also inhibit HDAC function by using TSA and then measure OVOL1 protein turnover rate. Experiments such as CHX-chase assays will help determine whether stability of OVOL1 is important in its function.

Regulating trophoblast proliferation and differentiation is critical for the maintenance of the fetal-maternal barrier in the placenta. Furthermore, OVOL1 is not only important in placental-related diseases, but it is also important in regulating cell growth in cancers involving many types of epithelial cells. OVOL1 acts downstream of signaling pathways involved in regulating the mesenchymal-to-epithelial transition (MET) in prostate and breast cancer, and squamous cell carcinoma. OVOL1 represses ZEB1 (an inducer in epithelial to mesenchymal transition), and works with transcription factors such as AP1, STAT1, STAT3 and NFKB1 to regulate MET [21,22]. Overall, our findings validate our hypothesis that OVOL1 regulates cellular proliferation through its interaction with class II HDACs (diagram of hypothetical model explained in Fig 2.11). Our findings in yeast help us understand the underlying mechanisms through which OVOL1 acts and adds to the repertoire of work done in many other model organisms. Future work is needed to better delineate OVOL1 interactions and transcription regulation in mammalian epithelial development. These discoveries will provide insight into the mechanisms through which OVOL1 acts to repress gene expression and enhance

our fundamental understanding of placental biology and in producing therapeutic targets for cancer/disease treatments.

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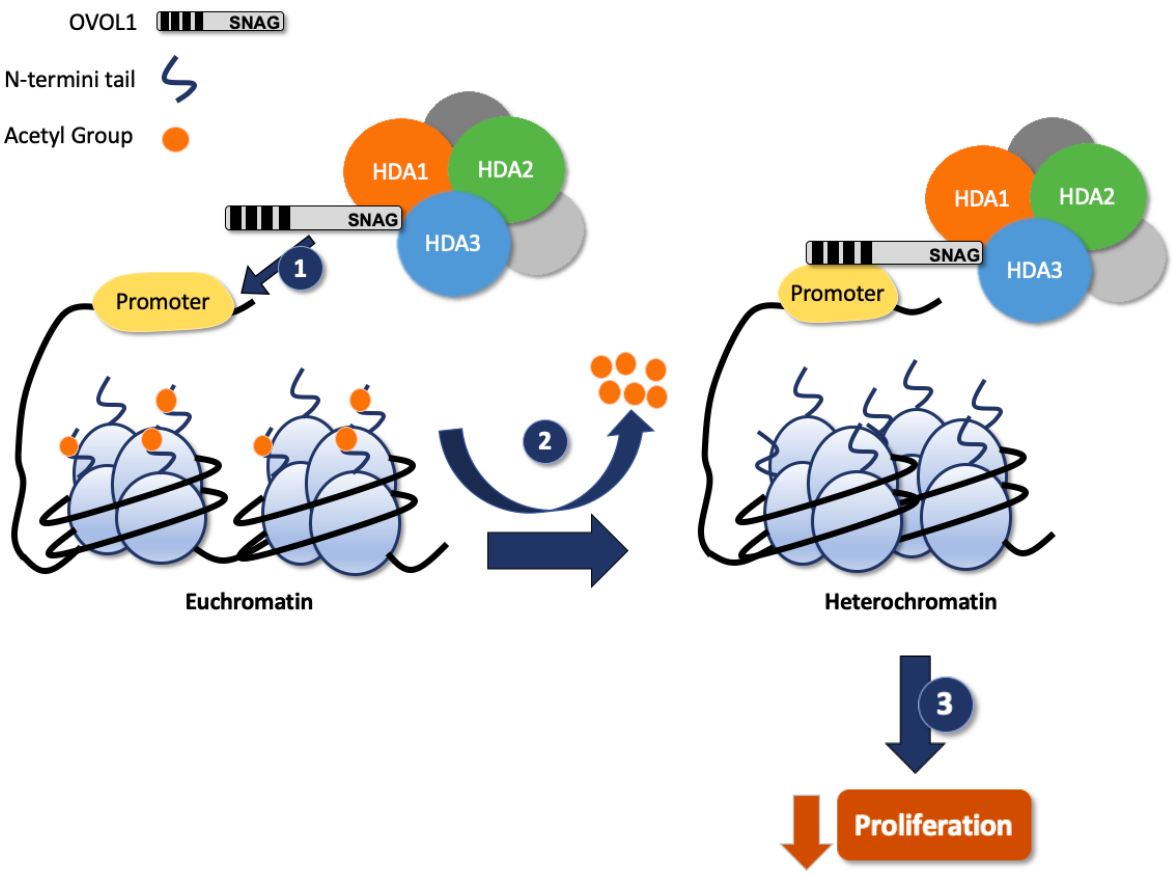

Figure 2.11 Hypothetical Model Underlying OVOL1 Transcriptional Regulation. We hypothesize that OVOL1 is able to repress genes necessary for cellular proliferation. OVOL1 recruits class II HDACs through its SNAG domain (1). Using OVOL1's zinc finger region, it is able to bind to DNA thereby causing deacetylation and remodelling the chromatin (2) into a more condensed state. This state prevents transcription factors and other transcription-driving machinery from accessing the promoter of target genes, thereby causing gene repression (3).

Curriculum Vitae

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Poster: Albakri, MB., Dhaliwal, H., Renaud., SJ., and Lajoie, P. Uncovering the Role of OVOL1 in Cell Growth Regulation using *Saccharomyces cerevisiae:* Implications for Placental Homeostasis.
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