CHARACTERIZATION OF EPIGENETIC MECHANISMS INVOLVED IN EMBRYO IMPLANTATION

(DNA METHYLATION GOVERNED EXPRESSION OF THE NOVEL TEAD4 PROMOTER)

Ph.D. Thesis

Shima Rashidiani

Supervisor:

Dr. Tibor A. Rauch PhD.

Interdisciplinary Doctoral School of Medicine

Prof. Dr. Ferenc Gallyas PhD, DSc.

Leader of the program and the doctoral school

University of Pécs

Medical School

Department of Biochemistry and Medical Chemistry

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ABBREVIATIONS

ART - Assisted reproductive technology
DBD - DNA-binding domain
DNMT - DNA methyltransferase
ER - Estrogen receptor
ES - Embryonic stem
EWAS - Epigenome-wide association studies
FBS - Fetal bovine serum
HAT - Histone acetyltransferase
HDAC - histone deacetylase
HMTs - Histone methyltransferse
ICM Cells - inner cell mass
iPSCs - Induced pluripotent stem cells
LSD1- lysine-specific demethylase
MLLI - Mixed lineage leukemia
PGCs - Primordial germ cells
PRB - Progesterone receptor B
PTMs - Post-translational modifications
PRC2 - Polycomb repressive complex 2
SAM - S-Adenosyl-methionine
TAZ -Transcriptional coactivator with PDZ-binding motif
TEADs -Transcriptional enhanced associated domain transcription factors
TFs - transcription factors
TNBC - Triple negative breast cancer
TrxG - Trithorax group
YAP - Yes associated protein
YBD - Yap binding domain

1. INTRODUCTION

One in seven couples worldwide suffers from infertility, and implantation failure can be a leading cause of involuntary childlessness (1). Human embryo implantation is an essential spatiotemporal process for establishing a successful pregnancy. This process contains three different phases including apposition, attachment, and invasion. Embryo implantation needs a high-quality embryo and a receptive endometrium to occur (2). The importance of epigenetics in endometrial receptivity remains an active area of research in embryo implantation. Several gene expression profiles are participated in decidualization and implantation. Recently, it has been demonstrated that epigenetic modifications, including DNA methylation, histone-modification, and microRNAs are involved in targeting this expression (2). The characterization of epigenetic mechanisms and transcription factors involved in embryo implantation in the endometrium may contribute to a better understanding of this pivotal developmental process and pave the way for future treatments. This work describes the discovery of an alternative isoform of the transcription factor TEAD4, which is the earliest gene required for trophectoderm lineage differentiation and subsequent embryonic implantation and processes. The investigation also examined whether epigenetic mechanisms such as DNA methylation play a role in regulating the expression of the new TEAD4 isoform in specific tissues, including the umbilical cord and placenta.

1.1. Epigenetics

An increasing body of research shows that in addition to the inherited genetic architecture (i.e., genomic DNA) various environmental factors contribute significantly to the etiology of disease. Epigenetic mechanisms respond to external stimuli and act as a bridge between the environment and the DNA that carries genetic information. Epigenetic mechanisms regulate gene expression and influence cellular activity by interpreting genetic information. Alterations in their profile can have significant effects. Overall, epigenetic mechanisms increase the complexity of most disorders by providing subtle contributions to their manifestation (3). Although there is controversy regarding the involvement of genetic and epigenetic factors in disease etiology, it is becoming increasingly clear that these two systems interact and are ultimately responsible for the development of the most complex diseases. There is no set definition for epigenetics, but it is generally considered to be the study of heritable changes in gene function that do not involve changes in the primary DNA sequence (4). Epigenetics was originally focused on DNA

methylation and various histone modifications but has recently broadened to include non-coding RNAs. *Ab ovo*, every cell in the body inherits the same genetic information (5). What makes each cell unique is that different sets of genes are turned on and off during ontogenesis. Epigenetics, in a broad sense, is a bridge between genotype and phenotype—a phenomenon that changes the final outcome of a locus or chromosome without changing the underlying DNA sequence. For example, even though the vast majority of cells in a multicellular organism share an identical genotype, organismal development generates a diversity of cell types with disparate, yet stable, profiles of gene expression and distinct cellular functions. Thus, cellular differentiation may be considered an epigenetic phenomenon, largely governed by changes in what Waddington described as the "epigenetic landscape" rather than alterations in genetic inheritance (6). More specifically, epigenetics may be defined as the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA (7). Epigenetic mechanisms define the proper nuclear environment for cell-specific gene expression and are responsible for cellular memory, i.e., the maintenance and transmission of cell-specific gene expression patterns to daughter cells. Epigenetic factors can deposit, interpret, and erase epigenetic information, and in this sense can be divided into different functional groups: epigenetic "***writers***" or enzymes that modify DNA and histones; epigenetic "***readers***" with specific protein domains that recognize DNA or histone marks; and epigenetic "***erasers***" that can delete the existing signals to make room for new modifications.

1.2. Epigenetic Writers, Readers, and Erasures

Modifications of DNA and histone proteins occur by adding several chemical groups utilizing numerous enzymes. Although numerous modifications are possible, it has been focused on the two most widely studied epigenetic alterations via, methylation and acetylation. Both DNA and histone proteins are susceptible to methylation, while acetylation is associated only with histones. By altering transcriptional activation or repression, these two modifications frequently govern the gene expression pattern in a cell. The epigenetic writers include DNA methyltransferases, histone lysine methyltransferases, protein arginine methyltransferases, and histone acetyltransferases (8). A wide range of epigenetic modifications, which are intricately laid down by specialized enzymes known as epigenetic writers, must be identified by other proteins within the cell to influence gene expression and cellular function. To facilitate this recognition process, mammalian cells have evolved several protein domains specifically designed to attach to these modifications. These

domains are collectively referred to as epigenetic readers. Many chromatin modifiers act as epigenetic readers and are crucial for the epigenetic regulation mechanism. They are equipped with unique domains that enable them to detect and bind to various covalent modifications present on DNA and histones, thereby interpreting the epigenetic marks and translating them into active biological responses. This dynamic interplay between writers, readers, and the resulting epigenetic landscape plays a pivotal role in regulating gene activity without altering the underlying DNA sequence (9). The epigenetic marks settled in the form of post-translational modifications on histones and covalent modifications on DNA are temporary. These marks can be removed depending on the requirement of the cell to modify the expression states of the locus. To establish this, a group of enzymes known as erasers are available that oppose the activity of the writers. The erasers mediate the removal of epigenetic marks, which relieves its effect on transcription, leading into the modulation of gene expression (10). It is advisable to introduce these functionally diverse protein factors according to the types of corresponding epigenetic modifications. Accordingly, we can discuss DNA methylation and histone modification related to writers, readers, and erasers.

1.2.1. DNA methylation-associated epigenetic factors

DNA methylation is one of the most studied epigenetic modifications and its processes are well characterized (Figure 1).

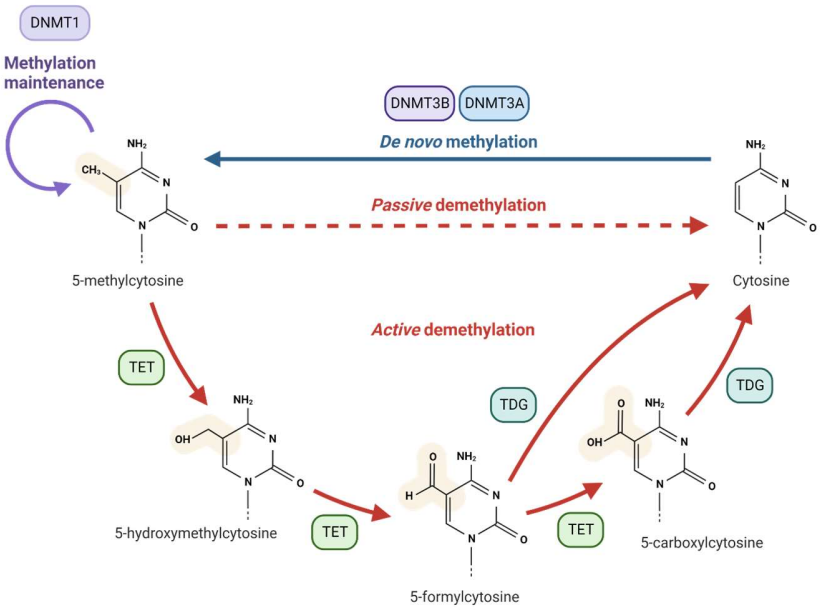

Figure 1. Summary of biochemical pathways of DNA methylation (11).

1.2.1.1. DNA methylation and its writers

DNA methylation, the most extensively studied epigenetic mark, plays a critical role in numerous epigenetic processes, including genomic imprinting, transposon silencing, X-chromosome inactivation, and gene silencing. It is also involved in vital biological functions such as early embryogenesis, stem cell differentiation, regulation of neuronal development, and oncogenesis (12,13). DNA methylation involves the addition of a methyl group to the 5th carbon of the cytosine pyrimidine ring, forming 5-methylcytosine (5mC). In general, CpG dinucleotides are predominantly methylated in mammals, correlating with transcriptional inhibition at CpG island (CGI) promoters. Interestingly, DNA methylation within gene bodies has been associated with active transcription(14). Recent research has shown that DNA methylation can also trigger transcriptional activation, a phenomenon that is particularly pronounced in oocytes, germ cells, and pluripotent cells. DNA methylation is known to repress gene expression by inhibiting the binding of certain transcriptional activators and/or recruiting methyl-binding proteins with repressive functions (15).

DNA methyltransferases (DNMTs) are a family of enzymes responsible for writing and maintaining DNA methylation. These enzymes specifically recognize cytosines and transfer a methyl group from S-adenosylmethionine (SAM) to target DNA sequences. DNA methylation occurs at the C5 position of CpG dinucleotides and is catalyzed by two major classes of enzymes - *maintenance methylation* and *de novo methylation*. DNMT1 is the proposed maintenance methyltransferase responsible for copying DNA methylation patterns to the daughter strands during DNA replication. Without DNMT1, unmethylated daughter strands would be produced, leading to *passive* demethylation and genome instability. Mouse knock out models with both copies of DNMT1 deleted are embryonic lethal. DNMT3a and DNMT3b are considered to be the *de novo* methyltransferases that establish DNA methylation patterns during the early development (16).

1.2.1.2. Readers of DNA methylation

Understanding the function and significance of DNA methylation in gene expression and cellular differentiation necessitates a detailed look at the proteins that read these methylated signals. These proteins, broadly classified as "readers" of DNA methylation, are crucial for translating epigenetic marks into biological outcomes, primarily gene repression but also activation in certain contexts. The primary group of proteins involved in reading methylated DNA are the methyl-CpG binding

domain (MBD) proteins. This family includes several key members: MeCP2, MBD1, MBD2, MBD3, and MBD4. Each has unique binding characteristics and biological functions but commonly serves to recognize and bind to methylated CpG dinucleotides within the DNA. MeCP2 is perhaps the most well-studied due to its clinical significance; mutations in the MeCP2 gene are the primary cause of Rett syndrome, a severe neurological disorder. Functionally, MeCP2 binds to methylated DNA and recruits other chromatin remodeling proteins that contribute to chromatin compaction, leading to gene silencing. This protein plays a particularly pivotal role in neuronal cells, where it modulates the expression of various genes crucial for normal function (17). MBD1 is implicated in maintaining genome stability and regulating gene transcription through its interaction with chromatin modifiers and corepressors. It preferentially binds to methylated DNA and recruits histone H3 lysine 9 (H3K9) methyltransferases, reinforcing the transcriptionally inactive state of chromatin (18). MBD2 and MBD3 do not bind methylated DNA as strongly as MeCP2 or MBD1 but are part of the NuRD (nucleosome remodeling and deacetylase) complex, which plays a significant role in chromatin remodeling and transcriptional repression. MBD2, in particular, has been shown to be essential in mediating the effects of DNA methylation on gene silencing (19). MBD4 has a slightly different role, mainly involved in DNA repair. It binds to methylated DNA at sites of cytosine deamination, recognizing thymine-guanine mismatches and initiating repair processes to prevent mutations, thus maintaining genomic integrity (20). In addition to the MBD family, another significant reader of methylated DNA is UHRF1 (Ubiquitin-like with PHD and RING Finger domains 1), which plays a key role in DNA methylation maintenance during DNA replication. UHRF1 recognizes hemimethylated DNA and recruits DNMT1 to these sites, ensuring the newly synthesized DNA strand acquires the same methylation pattern as the parent strand (21). These methyl-CpG binding proteins underscore a complex regulatory system wherein DNA methylation is intricately linked to other histone modifications and chromatin remodeling activities. This interplay is crucial for the dynamic regulation of gene expression across different tissues and developmental stages, and disruptions in these processes are often associated with diseases.

1.2.1.3. Erasures of DNA methylation

DNA methylation is a reversible modification, and the enzymes responsible for removing methyl groups from DNA—termed "erasers"—are crucial for dynamic changes in gene expression across various biological processes and developmental stages. The primary mechanisms of DNA

demethylation include passive demethylation and active demethylation, each facilitated by distinct enzymatic activities. Passive demethylation occurs during DNA replication. This process involves the dilution of methyl marks due to the absence or reduced activity of DNA methyltransferases (DNMTs), particularly DNMT1, which fails to maintain methylation patterns after cell division. As a result, successive cell divisions lead to progressively less methylated DNA in the daughter cells, contributing to changes in gene expression necessary for cell differentiation and development. Active Demethylation, in contrast, is a more direct and immediate process, involving several key players, primarily the Ten-Eleven Translocation (TET) family of enzymes. These enzymes—TET1, TET2, and TET3—catalyze the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), which is an intermediate step toward full demethylation (figure 2). This conversion is crucial as 5-hmC is further processed either by further oxidation to form 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), which can be excised by thymine DNA glycosylase (TDG), and subsequently replaced with unmodified cytosine via the base excision repair (BER) pathway. TET Enzymes not only facilitate active demethylation but also serve as epigenetic markers in their own right. For instance, 5-hmC is enriched in gene bodies and at enhancers, particularly in the brain and embryonic stem cells, suggesting a role in active transcription regulation and pluripotency (22). Moreover, the distribution and regulation of TET enzymes have significant implications in developmental biology and are associated with various cancers when mutated or dysregulated. The regulation of TET activity is complex and influenced by factors such as substrate availability, post-translational modifications, and interacting proteins. For example, the availability of alpha-ketoglutarate and oxygen, which are substrates and cofactors for TET enzymes, respectively, can affect their enzymatic activity, linking cellular metabolism to epigenetic regulation (23). The process of active demethylation is essential not only for normal development but also in response to environmental stimuli, enabling cells to rapidly alter their transcriptional landscape in reaction to external signals. Dysregulation of demethylation processes is linked to developmental abnormalities and various diseases, including neurodegenerative disorders and cancer.

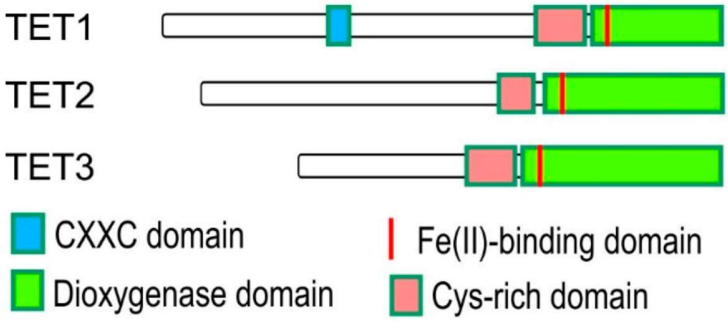

Figure 2. Erasure of DNA methylation. Domain structure of TET proteins. CXXC: cysteine-rich Zn2+ binding domain, Fe (II)-binding domain, Dioxygenase domain, Cys-rich domain (24).

1.2.2. Histone code-associated epigenetic factors

The histone code hypothesis posits that the post-translational modifications (PTMs) of histone proteins encapsulate a regulatory language that dictates chromatin architecture and gene expression. These PTMs, such as methylation, acetylation, phosphorylation, and ubiquitination, occur at specific residues on histone tails and serve as molecular signals that are interpreted by a suite of protein complexes. This epigenetic code modulates the accessibility of DNA to transcriptional machinery, thereby influencing gene activity in a cell-type and temporal-specific manner. Key to the functionality of this system are histone code-associated epigenetic factors, which are categorized into three primary roles: 'writers' that deposit PTMs, 'erasers' that remove these chemical groups, and 'readers' that recognize and bind to these modifications to effect downstream biological responses. Each class of these proteins is critical for the maintenance of the structural and transcriptional integrity of the genome. For example, histone acetyltransferases (HATs) add acetyl groups to lysine residues, enhancing transcriptional activity by reducing chromatin compaction. Conversely, histone deacetylases (HDACs) remove these acetyl groups, generally leading to a more condensed chromatin state and transcriptional repression. The specificity and combinatorial nature of histone PTMs create a complex layer of regulation that can lead to diverse biological outcomes. Dysregulation of these modifications has been implicated in a myriad of pathologies, including oncogenesis, where alterations in histone modification patterns can perpetuate malignant phenotypes. This thesis delves into the molecular mechanisms by which histone code-associated epigenetic factors govern chromatin dynamics and gene expression,

focusing on their roles in cellular differentiation and disease pathology, thereby enriching our understanding of epigenetic regulation in human health and disease (25).

1.2.2.1. Histone acetylation and deacetylation

Histone acetylation is a crucial process in chromatin remodeling and gene expression regulation. **Epigenetic code writers**, known as **histone acetyltransferases** (HATs), add acetyl groups to lysine residues on histone tails. This neutralizes their positive charge and reduces their affinity for the negatively charged DNA. This modification results in an open chromatin structure, which facilitates access to transcriptional machinery and promotes gene expression (26). Histone acetyltransferases (HATs) utilize acetyl CoA as a cofactor to add an acetyl group to the -amino group of lysine. This neutralizes the positive charge on lysine, weakens the histone-DNA interaction, and makes genes accessible (27). HATs are a heterogeneous group of proteins, with approximately 30 identified in humans thus far. They are largely categorized into two classes based on their subcellular location: Type A HATs, found in the nucleus, and Type B HATs, located in the cytoplasm. Type A and Type B HATs have distinct roles in histone acetylation (28). Type A HATs are involved in transcription-related histone acetylation in chromatin, while Type B HATs acetylate newly generated histones and affect the structure of the nucleosome (29).

The bromodomain is a conserved protein module present in various chromatin- and transcription-associated proteins. Proteins containing bromodomains can recognize acetylated lysine residues and act as **epigenetic reader**s by recruiting transcriptional coactivators or chromatin remodeling complexes to acetylated chromatin regions(30). For instance, the BET family of proteins possess tandem bromodomains that bind to acetylated histones, coupling chromatin acetylation to active transcriptional elongation (31). Bromodomains are classified into distinct subgroups, each with specific structural and functional characteristics. The bromodomain family functions as acetyl-lysine binding domains and are highly druggable, making them potential targets for epigenetic drug development (32).

Histone deacetylases (HDACs) are a family of **epigenetic erasures** that remove acetyl groups from lysine residues on histone tails, leading to a more compact chromatin structure and typically resulting in gene repression (33).The HDAC family is divided into different classes with distinct localization and functions. Class I HDACs are similar to the yeast RPD3 protein and are primarily nuclear, while class II HDACs are homologous to the yeast HDA1 protein and are found in both the nucleus and cytoplasm. In addition, class III HDACs are NAD-dependent enzymes

known as sirtuins, which are structurally distinct from the other classes (27).Dysregulation of HDAC activity has been implicated in cancer, neurodegenerative and cardiovascular diseases, highlighting their importance in maintaining cellular homeostasis. The acetylation and deacetylation processes controlled by histone acetyltransferases (HATs) and HDACs, respectively, have been extensively studied for their impact on chromatin function and gene expression (34).

1.2.2.2. Histone methylation and demethylation

In the epigenetic regulatory environment, histone methylation has a bifunctional role, acting to modulate gene transcription in a manner distinct from the effects observed with histone acetylation (35,36). This bifunctionality depends on the specific lysine residues that are methylated and the epigenetic context of these modifications. **Histone methyltransferases** (HMTs), **histone code writers**, such as EZH2, a component of the Polycomb repressive complex 2, are responsible for the trimethylation of H3K27, a modification synonymous with transcriptional silencing. On the other hand, methylation at H3K4 by the enzyme MLL1 (mixed lineage leukemia 1) correlates with transcriptional activation. These examples illustrate the intricate regulatory mechanisms governing gene expression (37).Unlike histone acetylation, which consistently signals transcriptional activation, histone methylation can either inhibit or promote gene expression (38). This modification targets lysines on histones H3 and H4 and can manifest in mono-, di-, or tri-methylated states. These methylations do not alter the histone charge but instead attract proteins that either silence or regulate gene activity, which is critical for cellular differentiation and lineage specification (35). Specifically, H3K4me3 prevents long-term gene repression by competing with transcriptional repressors or inhibiting DNA methylation. This highlights the complex and dynamic interplay of histone modifications in the epigenetic regulation of gene expression (37,39).

Chromodomain containing proteins are a family of **epigenetic readers** that primarily bind to methylated lysine residues on histones. The HP1 family of proteins, for example, contains chromodomains that specifically recognize the methylated histone H3 at lysine 9 (H3K9me), which is a marker for heterochromatin and transcriptional silencing. This interaction is crucial for the formation and maintenance of heterochromatin, contributing to genomic stability and gene regulation. Chromodomains are small domains, typically around 55 amino acids in length, interact with a range of nuclear proteins and are involved in diverse functions such as chromatin targeting, nucleosome mobilization, and regulation of gene expression. The ability of chromodomains to

discriminate between repressive methyl-lysine marks in histone H3 has been well-documented. Initially, histone methylation was thought to be an irreversible process due to its half-life being roughly equal to the half-life of histone. **Histone demethylases** (KDMs) are **epigenetic erasures** that mediate the removal of methyl groups from the lysine residues of histones. The discovery of KDMs revealed that histone lysine methylation is a reversible modification. Several dozen KDMs have been identified, each possessing unique motifs and functional domains for enzymatic activity, suggesting a complex gene regulatory function (40).

1.3. Epigenetic aspects of the preimplantation

Preimplantation development involves the fertilization of the oocyte by the sperm through the implantation of the hatched blastocyst into the endometrium. After the fertilization of the oocyte, the single-cell zygote is formed, which undergoes several rounds of cleavage without increasing the whole embryo volume(41). As the embryo reaches the eight-cell stage, polarization allows compaction, which provides a foundation for establishing distinct cell lineages: the trophectoderm (TE) and inner cell mass (ICM). These cell lineages evolve over subsequent asymmetric cleavage divisions, continuing through the formation of the blastocoel cavity. Cavitation results in the full expansion of the blastocyst, which hatches from its surrounding zona pellucid layer before implanting into the endometrium(42). Preimplantation embryo development extends from fertilization to implantation and progresses through several key stages: fertilization, cell cleavage, morula formation, and finally, blastocyst formation. Understanding these stages and their regulatory molecular mechanisms is crucial for the fields of reproductive biology and regenerative medicine (43). Identifying the global patterns of gene, RNA, and protein expression in early embryos is essential to understand these regulatory processes. Initial research efforts utilized methods such as comparative electrophoretic analysis with radiolabeled tyrosine and lysine to study protein expression patterns, while RNA expression was examined through cDNA library analysis (44). Researchers introduced enhanced or new techniques as the field progressed, including PCR-based differential display and subtractive cDNA library construction. The advent of microarray technology marked a significant advancement, rapidly becoming a dominant method that offers detailed and comprehensive data on global expression patterns, particularly gene expression profiles (45). Researchers have identified distinct phases of preimplantation development based on gene expression changes in mouse embryos: Phase I from fertilization to the 2-cell stage, Phase II from the 4-cell to the 8-cell stage, and Phase III from the 8-cell stage to

the blastocyst. Phases I and II are characterized by zygotic genome activation (ZGA) and mid-preimplantation gene activation, respectively. During ZGA, certain proteins formed during oogenesis persist post-fertilization and play roles in guiding subsequent developmental stages (46). Both genetic and epigenetic factors influence preimplantation embryo development. Researchers have delved into how epigenetic profiles interact with genetic information during embryogenesis, highlighting their significant roles in inducing phenotypic changes without altering the DNA sequence (47,48). Epigenetic mechanisms play key roles in this process, including DNA methylation, histone modifications, chromatin remodelling, and various types of RNA interference (RNAi). Each cell acquires a unique epigenetic signature that evolves as it undergoes specific processes such as differentiation or fertilization. Initial research primarily explored the impact of DNA methylation and how histone modifications influence embryo development during these early stages (49). Subsequent studies have broadened our understanding by incorporating aspects of chromatin organization, such as core histone variants, into the framework of epigenetic regulation. This complexity not only adds depth to our understanding of genetic regulation but also enriches life's mysterious and beautiful beginnings (50,51).

1.3.1. DNA methylation in preimplantation

DNA methylation is an epigenetic modification involving transferring a methyl group from the coenzyme S-adenosyl-L-methionine to cytosine residues in CpG dinucleotides. This process is critical for regulating chromatin structure and gene expression across various developmental stages, including gene imprinting, X-chromosome inactivation, and embryogenesis (9). Various techniques such as sodium bisulfate DNA modification, methylation-sensitive restriction enzyme digestion, and PCR or hybridization methods like Southern blotting and microarrays are employed to assess DNA methylation. More recently, attention has shifted towards comprehensive genome-wide DNA methylation profiling, utilizing techniques such as microarrays, high-performance liquid chromatography (HPLC), and restriction landmark genomic scanning (RLGS) (52). DNA methyltransferases (Dnmts), which are essential for establishing and maintaining DNA methylation, come in three types: Dnmt1, Dnmt2, and Dnmt3 (which includes Dnmt3a, Dnmt3b, and Dnmt3l) (53). Dnmt1 is particularly significant in maintaining methylation patterns during DNA replication, predominantly targeting hemimethylated DNA. Dnmt3a and Dnmt3b catalyze de novo methylation but have differing substrate preferences, while Dnmt3l, despite lacking enzymatic activity, plays a critical regulatory role. In preimplantation embryogenesis, dynamic

shifts in DNA methylation occur. After fertilization, the paternal and maternal pronuclei form, undergoing active and passive demethylation, respectively. As the embryo progresses, de novo methylation occurs, which is crucial for the first cell differentiation and silencing genes that maintain pluripotency. Variability in methylation patterns, even among laboratory mice, and contradictory findings in other mammals suggest that methylation's developmental roles may be species-specific (54,55). Despite global fluctuations in methylation levels, the methylation status of imprinted genes remains consistent. Studies have shown that Dnmt1 can maintain methylation at most imprinted loci without the aid of Dnmt3a and Dnmt3b. Additionally, the isoform Dnmt1s is associated with the nucleus throughout preimplantation development, helping to maintain methylation at specific genomic regions (56). Methylation also varies beyond global levels; Dnmt1o, another Dnmt1 isoform primarily expressed during oogenesis and early preimplantation, shows stage-specific methylation changes. The analysis of mRNA expression patterns of methyltransferases and related proteins indicates divergent epigenetic profiles within a single embryo. This complexity necessitates detailed single-cell methylation profiling to discern cellular fates and contributes to understanding the intricate process of DNA methylation reprogramming during early development (57). DNA methylation is thus a fundamental and dynamic epigenetic modification essential for preimplantation embryonic development, affecting both global and locus-specific gene expression and highlighting the necessity for detailed epigenetic profiling at the single-cell level (58).

1.3.2. Histone modifications and chromatin remodeling in preimplantation

Histone modification is a crucial covalent modification that regulates gene expression through processes including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. These modifications are grouped into smaller chemical modifications (acetylation, methylation, phosphorylation) and larger peptide modifications (ubiquitylation and sumoylation)(59). While acetylation and methylation are more widespread and well-studied due to their common occurrence, other types of modifications also play specific roles during preimplantation development. Histone modifications interact with DNA methylation to enhance epigenetic regulation further. For example, the histone methyltransferase Suv39h1 facilitates H3K9 trimethylation, which is crucial for attracting Dnmt3b-dependent DNA methylation to pericentromeric repeats(60). Similarly, DNA methylation helps recruit methyl-binding domain proteins (MBDs) and assemble histone deacetylase complexes (e.g., MeCP1 and Mi2/NuRD),

contributing to the complexity of epigenetic regulation. These interactions indicate that histone modifications can be more dynamic and adaptable than DNA methylation's relatively stable silencing effect (61,62). Throughout preimplantation development, histone modifications continuously change. From fertilization to syngamy, modifications such as acetylated lysine (H4ac), and methylated histones (H3K4me, H3K9me2/3, H3K27me1, H4K20me3) are prominent in the female pronucleus, indicative of active and repressive chromatin states (59). The male pronucleus undergoes similar transformations, with initial acetylation being replaced by methylation. These modifications facilitate critical developments in gene expression regulation. Histone modifications are particularly important as they can be stage- and cell-type-specific, acting as switches that finely tune gene expression. For example, key genes such as Oct4 and Nanog are progressively silenced during cell differentiation through specific histone methylation patterns. In the blastocyst stage, the inner cell mass (ICM) and trophectoderm display different histone modification profiles, correlating with their divergent developmental pathways (63). Enzymes involved in histone modifications, such as histone methyltransferases (HMTases like G9a, ESET, and Suv39h) and histone deacetylases (HDACs such as HDAC1, HDAC2, and HDAC3), play pivotal roles. These enzymes not only modify histones but also facilitate the formation of functional chromatin structures essential for maintaining cellular identity and viability (64,65). Furthermore, ATP-dependent chromatin remodeling complexes (e.g., SWI/SNF, ISWI, and Mi-2/NuRD) modify histone-DNA interactions, enhancing the accessibility of DNA to transcription factors and other regulatory proteins. These complexes are integral during early embryonic stages, particularly during zygotic genome activation, ensuring that gene expression is accurately initiated (66).

1.4. TEAD family genes

The TEAD protein family consists of four distinct members, TEAD1-4, encoded by four separate genes, and expressed in almost every tissue type in mammals. The N-terminus domain binds with DNA fragments like 5′-GGAATG-3′, which are present in the SV40 enhancer and the promoter regions of TEAD target genes. The C-terminus functions as a transactivation domain for its recruitments of transcriptional coactivators. TEAD proteins alone are incapable of inducing genes expression and need additional coactivators to achieve their transcriptional potential. Coactivators cannot directly bind to the DNA, but they can bind with transcription factors to activate the transcription process. Several coactivator candidates for TEADs have been identified including

YAP and its paralog TAZ, vgll proteins, and the p160 family of nuclear receptor coactivators (67). TEAD1 is crucial for heart muscle development, promoting the expression of cardiac-specific genes(68). T\he role of TEAD2 remains somewhat elusive, but it is thought to be involved in regulating gene expression during brain development(69). TEAD4 is primarily associated with embryo implantation. The exact function of TEAD3 is still under investigation. Remarkably, almost all tissues express at least one TEAD gene, with some expressing all four(70). These four TEAD proteins share a similarity range of 61% to 73%. They are structured with a DNA-binding domain (DBD) at the N-terminus, consisting of about 80-90 amino acids, and a YAP/TAZ/VgLL binding domain (YBD) at the C-terminus, spanning approximately 220 amino acids (see Figure 2). The two domains are connected by a segment of approximately 90 to 100 amino acids, which exhibits limited homology among the four isoforms (70).

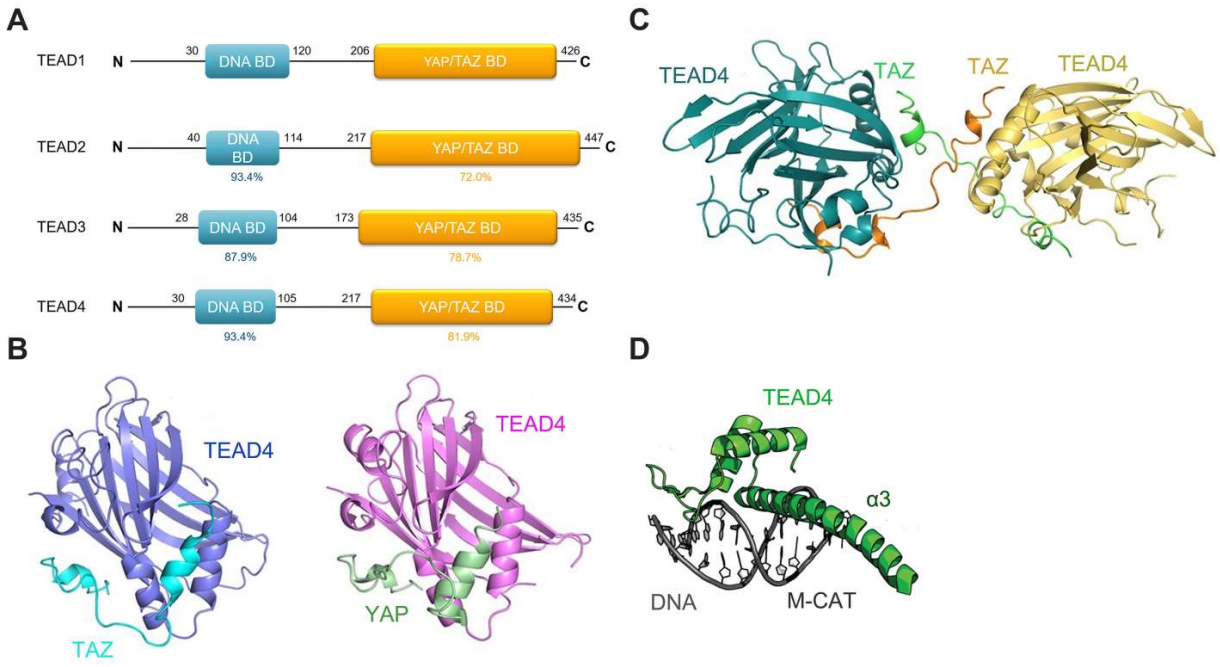

Figure 3. Domain architecture of four TEADs. (A) The overall structure of TEADs. TEADs consist of a TEA DNA binding domain (blue) and a YAP/TAZ binding domain (yellow). The percent represents the identity for each domain of TEADs compared to that of TEAD1. (B) YAP binds to TEAD4 via two short helixes and an extended loop containing the PXXΦP motif. TAZ interacts with TEAD4 in a similar manner to the binding of TEAD4-YAP. (C) Two TAZ molecules straddle two TEAD molecules to form a heterotetramer. (D) The α3 helix of the TEA domain (green) binds to the M-CAT DNA duplex (grey) (71).

1.5. TEAD4 protein and hipo signaling

TEAD transcription factors are key mediators of the Hippo signaling pathway. Their activity is modulated through interactions with nuclear coactivators, which are broadly categorized into three groups: (1) YAP (yes associated protein) and its paralog TAZ (transcriptional coactivator with PDZ-binding motif, also known as WWTR1), (2) VgLLs, and (3) p160s proteins(72)The Hippo signaling pathway is a regulator of organ size that operates through a core kinase cascade(73). YAP or TAZ, its downstream effectors, are located in the nucleus when unphosphorylated and relocate to the cytoplasm upon phosphorylation (Figure 4).

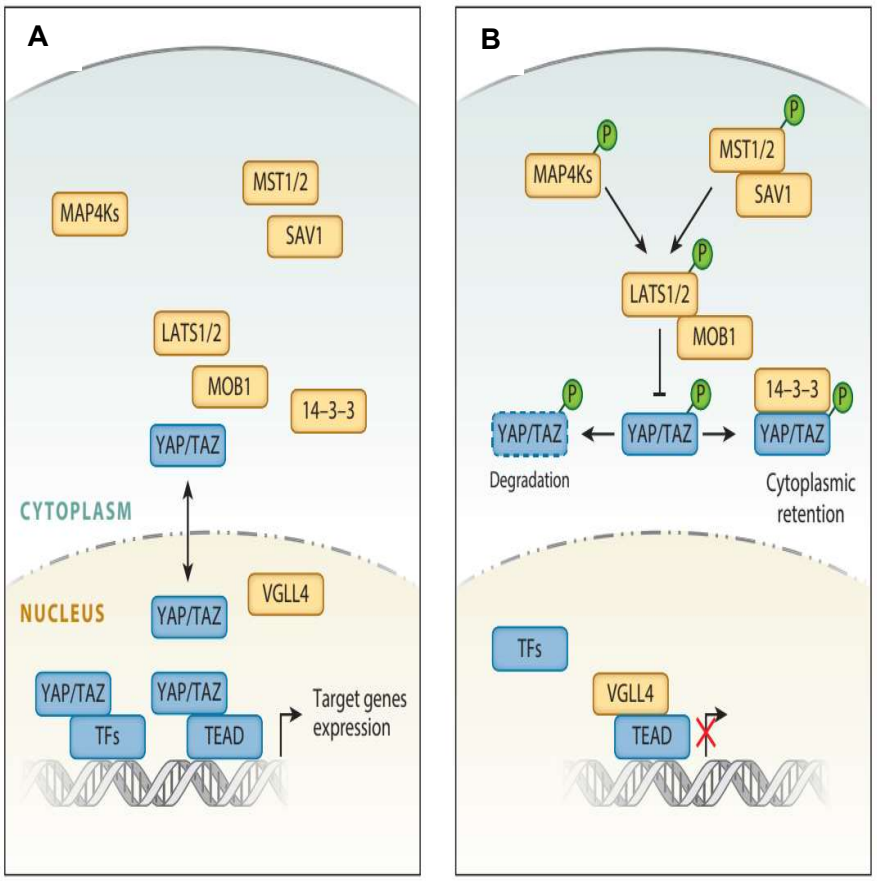

Figure 4: Hippo signaling controls embryonic cell fates. Core components of the Hippo pathway in mammalian cells. The Hippo pathway activity controls the dynamic localization of YAP/TAZ between nucleus and cytoplasm. (a) When the Hippo pathway is OFF, YAP/TAZ are dephosphorylated and accumulate in the nucleus, where they bind with TEADs and possibly other transcription factors (TFs) to induce gene transcription. (b) When the Hippo pathway is ON, the active LATS kinases phosphorylate YAP/TAZ, resulting in their binding to 14–3–3 and cytoplasmic retention as well as degradation. However, the Hippo pathway does not behave

digitally only in ON or OFF status. YAP localization can be partially cytoplasmic and partially nuclear depending on the relative activities of LATS kinase and the opposing phosphatase for YAP. The VGLL4 competes with YAP/TAZ in binding to TEAD and represses the target gene expression (76).

In their phosphorylated form, YAP and TAZ undergo degradation via the ubiquitin/proteasomal pathway. When unphosphorylated, YAP/TAZ move to the nucleus and activate various nuclear transcription factors, including TEADs (74,75). There is increasing evidence that some key components of the Hippo-YAP pathway are tightly regulated at the RNA level by alternative splicing, an established mechanism for increasing the coding capacity of the human genome (75). For instance, YAP has eight splicing isoforms with different internal sequences. The role of these isoforms has not been fully elucidated. As TEAD4 is a crucial component of Hippo signaling, it is hypothesized that additional isoforms may exist and play a biologically relevant role in this process (77). TEAD4 plays a critical role in the Hippo signaling pathway. The regulation of TEAD4 in this pathway depends on the phosphorylation status of its coactivators, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (78). When the Hippo signaling is activated, YAP and TAZ are phosphorylated, leading to their retention and degradation in the cytoplasm. In an inactive state, YAP and TAZ migrate to the nucleus and bind with TEAD4, enhancing its transcriptional activity. This regulation is crucial for controlling cell proliferation, organ size, and tissue homeostasis. TEAD4's role is also observed in gastric cancer, where it is modulated by HOXB13 within the Hippo pathway, affecting the proliferation, migration, invasion, and apoptosis of gastric cancer cells (79,80). TEAD4 plays a crucial role in the YAP-TAZ signaling pathway, promoting cancer cell proliferation in colorectal cancer. Additionally, TEAD4 is a key player in embryonic development and pluripotency, forming part of the transcriptional network that governs pluripotency in embryonic stem cells (81). It collaborates with core pluripotency factors, such as Oct4, Sox2, and Nanog, to regulate genes that are essential for maintaining embryonic stem cells in an undifferentiated state (82). This interaction emphasizes the significance of TEAD4 in early developmental stages and cellular differentiation. TEAD4 also has a role in the Wnt signaling pathway, which is key for cell fate determination and maintaining tissue homeostasis. In certain contexts, TEAD4 can interact with β-catenin, an essential mediator in Wnt signaling, to control gene expression (83). This interplay between TEAD4 and Wnt signaling

underscores the protein's capacity to integrate various signaling pathways, facilitating complex cellular responses (15)

1.6. Tead4 and embryo implantation

During pre-implantation mouse development, embryos form blastocysts through the establishment of the first two cell lineages: the trophectoderm (TE), which gives rise to the placenta, and the inner cell mass (ICM), which will form the embryo proper. Differentiation of the TE is regulated by the transcription factor Caudal-related homeobox 2 (Cdx2), but the mechanisms that act upstream of Cdx2 expression remain unknown (84). The TEA domain family transcription factor, Tead4, is required for TE development. Studies have shown that Tead1, Tead2, and Tead4 are expressed in pre-implantation embryos, with at least Tead1 and Tead4 widely expressed in both TE and ICM lineages. However, Tead4 knockout (Tead4^-/-) embryos die at pre-implantation stages without forming the blastocoel. Although cell proliferation, adherens junctions, and cell polarity remain unaffected in Tead4$^{-/-}$ embryos, Cdx2 is weakly expressed at the morula stage and not expressed at later stages. Furthermore, no TE-specific genes, including Eomes and the Cdx2-independent gene Fgfr2, are detected in Tead4$^{-/-}$ embryos. Instead, the ICM-specific transcription factors Oct3/4 and Nanog are expressed in all blastomeres (Nishioka et al., 2008). Tead4$^{-/-}$ embryos also fail to differentiate into trophoblast giant cells when cultured in vitro, although embryonic stem (ES) cells with normal differentiation abilities can still be established from these embryos. These findings suggest that Tead4 has a distinct role from Tead1 and Tead2 in the specification of pre-implantation TE and that Tead4 is an early transcription factor required for the development of the trophectoderm lineage, including Cdx2 expression (85). Functional redundancy between TEAD1 and TEAD2 has been observed because TEAD1 and TEAD2 double-mutant embryos exhibit more severe defects than either TEAD1 or TEAD2 single-mutant embryos (86). However, TEAD4 inactivation in mice severely affects trophectoderm specification, leading to embryo implantation failure, which appears to be the primary function of TEAD4. Notably, embryos develop normally when TEAD4 function is disrupted after implantation (86). It was initially demonstrated that TEAD4 is not necessary for blastocoel formation when embryos are cultured in low oxygen concentrations. However, Tead4 knockout embryos cultured in the absence of glucose fail to initiate blastocoel formation, unlike wild-type embryos. This observation suggests that TEAD4's role during pre-implantation development is to establish energy homeostasis essential for transitioning from the morula to the blastocyst (87). TEAD4 also plays a crucial role in post-

implantation development by controlling trophoblast progenitor self-renewal and stemness within the placenta primordium. In post-implantation mouse embryos, TEAD4 is selectively expressed in trophoblast stem cell-like progenitor cells (TSPCs). The loss of Tead4 in post-implantation mouse TSPCs impairs self-renewal, leading to embryonic lethality before embryonic day 9.0 (E9.0), a developmental stage equivalent to the first trimester of human gestation. Both TEAD4 and its cofactor, Yes-associated protein 1 (YAP1), are specifically expressed in cytotrophoblast (CTB) progenitors of the first-trimester human placenta. Some unexplained recurrent pregnancy losses (idiopathic RPLs) have been associated with impaired TEAD4 expression in CTB progenitors. By establishing RPL patient-specific trophoblast stem cells (RPL-TSCs), researchers demonstrated that TEAD4 loss correlates with defective self-renewal in RPL-TSCs and restoring TEAD4 expression rescues this ability (88). Global gene expression (RNA-Seq) analysis of TEAD4-depleted murine TSCs, combined with ChIP-Seq data, has revealed that TEAD4 directly regulates various cell cycle regulators, including multiple Cyclins/CDKs. TEAD4-mediated regulation of Cyclins/CDKs in primary trophoblast progenitors is essential for their self-renewal and expansion. Thus, TEAD4 plays an essential role in trophoblast cell homeostasis during placental development, preventing placental insufficiency and ensuring successful pregnancy (88,89).

2. SPECIFIC AIMS

Transcriptional regulation plays a critical role in the implantation of an embryo into the endometrium of the uterus, a process that is essential for successful pregnancy. TEAD4, a transcription factor, plays a central role in orchestrating the intricate molecular events that are critical for successful implantation of the embryo into the endometrium of the uterus. It was known to regulate the expression of genes involved in cell adhesion, trophoblast invasion and endometrial receptivity. Later, TEAD4 was also found to be involved in post-implantation development and is critical for placental development, specifically the formation and function of the placental labyrinth. Dysregulation of TEAD4 can lead to abnormalities in placental structure and function, potentially resulting in pregnancy complications such as intrauterine growth restriction or pre-eclampsia. Accordingly, studying the epigenetic context of TEAD4 provides valuable insights into its role in development, reproduction, and disease, and offers potential avenues for both diagnostic and therapeutic advances in reproductive medicine. Therefore, we proposed the following objectives to gain more information on TEAD4 and its potential isoforms:

1) To explore the epigenetic landscape around the TEAD4 gene on human chromosome 12.
2) Search for potential new promoter regions for TEAD4 that could explain the complex function of TEAD4.

After careful epigenetic analysis, we realized that a new TEAD4 isoform of unknown function might be present and wanted to explore its presence in tissues:

3) Clone and sequence the mRNA encoding the new TEAD4 isoform.
4) To study the gene expression pattern of the new isoform.
5) Determine the subcellular localization of the new isoform
6) Cloned the alternative TEAD4 promoter and studied its behavior in transient expression studies.

After demonstrating that a new isoform is present in the placenta, we added two new objectives:

7) To demonstrate that the mRNA encoding the new isoform is translated in the placenta.
8) To investigate how DNA methylation might be involved in its expression.

In summary, the main goals of my thesis were to explore potential new TEAD4 isoforms, characterize their expression, and investigate how epigenetic mechanisms such as DNA methylation could be involved in these processes.

3. MATERIALS AND METHODS

3.1. Experimental models

3.1.1. Cell cultures

The cell lines (K562, HEK 293T, and glioblastoma) were cultured in DMEM medium with 10% 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., MA, USA) in T75 flasks, maintained in a controlled environment (37°C, 5% CO2), and the medium was refreshed every other day. Cells were seeded at 1 x 10^5 cells/mL and grown until reaching a density of 1 x 10^6 cells/mL before sub-culturing at 80% confluency.

3.1.2. Dye-exclusion assay

First, Trypan Blue was sterilized and filtered before using it in order to get rid of particles in the solution that would disturb the counting process. Next, the cells were diluted in Trypan Blue dye of an acid azo exclusion medium by preparing a 1:1 dilution of the cell suspension using a 0.4% Trypan Blue solution. Non-viable cells will be blue, viable cells will be unstained.

3.1.3. Cell freezing and recovery

As detailed previously, cells were grown to 80% confluence, trypsinized and then transferred to a 15-mL Falcon tube. To pellet the cells, centrifugation at 800 rpm for 5 minutes was performed. Aspirating the supernatant and resuspending the cells in 1 ml of pre-chilled freezing solution (90 %complete medium, 10% DMSO). The cells were then pipetted into a cryotube and immediately placed on ice. After approximately 24 hours at -80°C, the tubes were transported liquid nitrogen (-196°C). For cell recovery after freezing, frozen cells were thawed and plated in a 10 cm cell culture dish with new complete medium. After allowing the cells to adhere for one day, their medium was replaced with a fresh, complete medium.

3.1.4. Placental samples

Placentas were obtained at the Pecs University Hospital's, Department of Obstetrics and Gynecology. During pregnancy, all subjects signed an informed consent form to donate their placenta after birth. All samples were collected with the approval of the University of Pecs Ethics Board and in accordance with the World Medical Association's Code of Ethics (Declaration of Helsinki) for human experiments. The placenta used in this study were all from term or near-term pregnancies (36-41 weeks) with normal birth weight and no pregnancy complications. From shortly after birth until sampling, placentas were kept at 4°C. The fetal side of one placenta was

sampled from two different sites, each mid-distance between cord, and periphery. Each sample was divided in two, half was preserved in RNA later at -20°C prior to RNA extraction and the other half was used for DNA extraction.

3.2. Epigenomic data analysis

Chromatin immunoprecipitation-related histone mark and RNA polymerase II next generation sequencing (NGS)-related data sets (i.e., Chip-Seq data) were downloaded from the encyclopedia of DNA elements (ENCODE) database (90), which is a publicly available source of genomic/epigenomic data sets at University of California Santa Cruz.

3.3. RNA isolation

Total RNA was extracted from cell cultures using the Direct-zol RNA microprep kit (Zymo Research, Carlsbad, CA, USA) following the manufacturer's recommendations. RNA quantity and purity were measured using a NanoDrop ND-2000 spectrophotometer. RNA preparation from human samples was approved by the Ethics Committee of the University of Pécs (Code: 3648—PTE 2020, Epigenetic and Transcriptional Factors Involved in Placental Development). Additional total RNA samples used in this study were obtained from the FirstChoice® Human Total RNA Survey Panel (ThermoFisher Scientific, Waltham, MA, USA).

3.4. Rapid amplification of cDNA ends (5'RACE)

To identify our RNA transcript the (5′ RACE) was performed according to the following method(14).which contains two steps. In the first step, template switching reverse transcription reaction generates cDNAs with a universal sequence of choice, introduced by a template switching oligo (TSO), attached to the 3′ end of the cDNA (5′ end of the transcript). In the second step, the 5′ end of the transcript can be identified via PCR amplification with primers that are specific to the gene of interest and the TSO handle.

3.5. Nucleotide sequence analysis (Sanger sequencing)

Recombinant plasmids were purified from bacteria were sequenced (Sanger sequencing) at the Department of Medical Genetics (University of Pecs) and "DNA Blast" online software was used to identify the corresponding human genomic region in ENCODE database at the University of California Santa Cruz.

3.6. Cloning of the full length and truncated TEAD4 into eukaryotic expression vectors

As previously described (15) to investigate the subcellular localization of the two TEAD4 isoforms corresponding ORFs were *in vitro* synthesized (IDT, Coralville, IA, USA) and cloned in frame with a green fluorescent protein (EGFP) or a red fluorescent protein (monomeric RFP) coding mammalian expression vectors (i.e, pEGFP-Nl and pDsRFP-Nl). Proper in-frame fusions of the coding regions were verified by Sanger sequencing. Recombinant plasmids carrying full-length (TEAD4-FL-GFP) and the N-terminal-truncated (TEAD4-ΔN-RFP) TEAD4 isoforms were transformed then purified from bacteria and used in transient co-transfection studies.

3.7. Transient transfection and confocal fluorescent microscopy

HEK293T cells were plated 18-24 hours before transfection. Cell cultures were 60% confluent at the time of transfection. Plasmids were combined in a 1:1 ratio and 293Tran transfection reagent (OriGene Technologies, llE Rockville, MD, USA) was used for co-transfection. Cells were fixed with 4% paraformaldehyde 48 hours after the transfection. The nuclei were stained with 4',6-diamidino- 2-phenylindole, and slides were mounted with ProLong Antifade Mountain media (Thermo Fisher, Waltham, MA). Images were captured on a Zeiss LSM 700 Confocal Microscope and analyzed with Zen 2.0 software (Zeiss). Slides were investigated at magnification 80x.

3.8. Transient expression and Luciferase assay

10^4 HEK293 cells/well were plated in 12-well plates 24 hours before transfection. Transient transfections were performed in triplicates using GeneJuice@ Transfection Reagent (Merck, Darmstadt, Germany) in a 3:1 ratio according to the manufacturer's instructions. The medium was replaced the following day and plates were incubated for an additional 24 hours. Cells were harvested 48 hours after transfection, rinsed in lx PBS and lysed using the Dual-Luciferase® Reporter. Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence of the samples was determined using a FLUOstar-OPTIMA (BMG Labtech, Offenburg, Germany) luminometer. Protein concentration of the samples was determined using a Thermo Scientific™ NanoDrop™ One UV-Vis spectrophotometer (ThermoFisher Scientific Waltham, MA, USA). Luminescence values were normalized to the total protein content of the samples.

3.9. Western Blotting

Placental tissue samples were collected from the Obstetrics and Gynaecology Department at the University of Pecs. Tissue samples were placed on dry ice and homogenized with a Dounce homogenizer in M-Per mammalian protein extraction buffer (ThermoFisher Scientific) supplemented with protease (Roche) and phosphatase inhibitor (Sigma) cocktail. Protein lysates (40 μg per lane) were loaded onto 12% SDS-polyacrylamide gels, transferred to PVDF membranes (ThermoFisher Scientific). The transfer was conducted for 120 minutes at 85V. To detect our target protein on the PVDF membrane, the membrane was first blocked for 1 hour at room temperature in freshly prepared 1xPBS-T containing 5% nonfat dried milk (blocking buffer). Membranes were first treated with 5% milk to block nonspecific binding and then incubated with primary antibodies directed against TEAD4 (1:200 final dilution; Thermo Scientific) and β-Actin (1:1000 final dilution; Cell Signaling) overnight at 4°C. Species-specific horseradish peroxidase-conjugated secondary antibodies were applied at a 1:2000 final dilution (Cell Signaling). Immunocomplexes were detected using Immobilon ECL Ultra Western HRP Substrate (Merck) through the Syngene G:BOX for Chemiluminescence and Fluorescence imaging (Syngene). Analysis of the results was conducted using GeneSys software version 2.1 (Syngene), and Bio-Rad Precision Plus Protein™ Standards Kaleidoscope™ served as the molecular weight markers.

3.10. DNA Methylation Analysis—Bisulfite Sequencing

Genomic DNA was extracted from umbilical cord and placenta tissue samples obtained from the Department of Obstetrics and Gynaecology at the University of Pécs using the Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Ethics approval for genomic DNA preparation from human samples was granted by the Ethics Committee of the University of Pécs (Code: 3648—PTE 2020, Epigenetic and Transcriptional Factors Involved in Placental Development). The isolated DNA underwent RNase digestion and removal of cellular proteins by salt precipitation, followed by bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Primers (TABLE 1) for bisulfite sequencing (BS) were designed using MethPrimer software v.2.0. PCR-amplified promoter regions were cloned into the pDrive vector. Plasmids were isolated from 5 bacterial colonies, and the methylation status of CpG sites was determined by Sanger sequencing.

Table 1. The list of primers used for Oligonucleotides used in end-point PCR, cloning and DNA methylation (i.e., Bisulfite Sequencing) analysis.

Analysis	**Name**	**Forward/Reverse**	**Notes**
5'RACE	Anchor_F1	GACCACGCGTATCGATGTCGACTTTTT TTTTTTTTTTV	
	Anchor_F2	GTATCGATGTCGACTTTTTTTTTT	
	GenSpec_RT	ACTCAAACCCTGGCAG	Reverse transcription
	GenSpec_R1	CATGGGACGTTCCGGC	
	GenSpec_R2	AAGCTCCTTGCCAAAAC	
Gene expr.	TEAD4-C_F	TGAGCAGAGTTTCCAGGAGG	End-point PCR
	TEAD4-C_R	CCTTGCCAAAACCCTGAGAC	
	TEAD4-del_F	TCCCCTTGAGCACCTCTTAC	End-point PCR
	TEAD4-del_R	TCTCAGGGTTTTGGCAAGGA	
Promoter cloning	TEAD-PromC_oF	TCATGCACTTCCCTATCCCA	Nested PCR
	TEAD-PromC_oR	TGAGGGCTGGAATGAGATGC	
	TEAD-PromC_InF	aactcgagCCACCCAACACAAGGCTAAG	
	TEAD-PromC_InR	aaaagcttGGCTGGAATGAGATGCGC	
	TEAD-PromAlt_oF	TCTCTTTGGCAGGAGGGAAG	Nested PCR
	TEAD-PromAlt_oR	CAGGAGGGTAAGAGGTGCTC	
	TEAD-PromAlt_inF	aactcgagCAGTCTGCTTCCCTGAGTCA	
	TEAD-PromAlt_inR	aaaagcttGTTAATCTGCCGTGCTGCTT	
	Prom-Seq_F	gtacgggaggtacttggagcg	Sanger Seq
	Prom-Seq_R	ccacctcgatatgtgcatctgtaaaa	
Fusing cDNAs to FP	TEAD4-EGFP_F	aaaaagcttGCC**ATG**GAGGGCACGGC	TTG mutated to ATG to increase translation
	TEAD4-EGFP_R	aa*ggatcc*TTCTTTCACCAGCCTGTA	
	TEAD4-RFP_F	**aaaagcttATG**GCTGCCATGTCGTCTG	
	TEAD4-RFP_R	aa*ggatcc*TTCTTTCACCAGCCTGTA	
	EGFP-Seq_F	CCTCGCCCTCGCCGGA	Sanger Seq
	RFP-Seq_F	GCCCTCGTAGGGCTTGC	
DNA methylation anal.	BS_PromC_F	ATGGTATGTTATATGGATTTTAAAT	
	BS_PromC_R	CAAAAAAAACTACTCCCTTCCTAC	
	BS_PromAlt_F	TAGGTTTTGTTGGGATTTAGATATT	
	BS_PromAlt_R	CATCCCACACACTAAAAATAAACTC	
	T7	GCCAAGCTCTAATACGACTCACTATA GG	Sanger Seq
	Sp6	GCCATTTAGGTGACACTATAGAATAC	

3.11. Quantitative methylation-specific PCR (qMSP)

To optimize the qMSP approach and the newly designed promoter-specific primers, the TATA box binding protein (TBP) promoter was used as a negative control and the XIST promoter as a positive control for DNA methylation profile analysis. TBP promoter-specific qMSP C_t values were used to normalize qPCR data sets. CFX96 PCR machine (Bio-Rad, Hercules, CA, USA) was used for qMSP assays and CFX manager software was used for data processing.

3.12. Electrophoretic-Shift Essay (EMSA)

To explore and delineate the protein-DNA binding interactions linked to the TEAD4-ΔN promoter, we employed the LightShift EMSA kit (Thermo Scientific) in accordance with the recommended procedure. The biotin-labeled oligonucleotides detailed in Table S1 were utilized. Protein extracts for the EMSA analysis were obtained from HEK293 cell cultures at 80% confluency, using a method outlined previously (91).

Analysis	Name	Forward/Reverse	Notes
EMSA	TEAD4-ΔN-Bio_F	CCCTCTGGGAGGCGGAATGGGAGGCCGAGCTG	5'-biotyn
	TEAD4-ΔN_F	CCCTCTGGGAGGCGGAATGGGAGGCCGAGCTG	
	TEAD4-ΔN_R	CAGCTCGGCCTCCCATTCCGCCTCCCAGAGGG	
	TEAD4-ΔN-Mut_Bio_F	CCCTCTGGGAGGCGGACGGGGAGGCCGAGCTG	5'-biotyn
	TEAD4-ΔN-Mut_F	CCCTCTGGGAGGCGGACGGGGAGGCCGAGCTG	
	TEAD4-ΔN-Mut_R	CAGCTCGGCCTCCCCGTCCGCCTCCCAGAGGG	
	TEAD4-cons-F-Bio	CGGCGATGTGACCTGGAATGTGGCGTCCGTAT	5'-biotyn
	TEAD4-cons-F	CGGCGATGTGACCTGGAATGTGGCGTCCGTAT	
	TEAD4-cons-R	ATACGGACGCCACATTCCAGGTCACATCGCCG	

Table 2. Oligonucleotides for DNA-protein interaction studies (EMSAs). The corresponding complementary reverse oligos were annealed to the 5'-biotinylated oligos. In competition assays, un-biotinylated forward wereannealed and used in essays with varying molar concentrations.

3.13. Statistical analysis

The statistical analyses were carried out using the SPSS 18.0 software (Statistical Package for the Social Sciences; IBM, NY, US). The Student T-test was used to evaluate potential differences between the mean values in tests and controls. The *P*-values are derived from two-tailed tests and $P \geq 0.05$ was considered statistically significant.

4. RESULTS

4.1. Epigenetic profiles predict a new promoter in the 3rd intron of TEAD4 gene

Our initial hypothesis was that the TEAD4 gene may have different promoters and that a new transcript may be involved in the implantation of blastocysts into the endometrium. Specifically, distinct isoforms of TEAD4 may have unique functions and regulate trophoblast formation, ultimately affecting implantation. Numerous instances have been documented in which different promoters have been utilized in the context of cell-type-specific genetic and epigenetic decisions. To find additional promoters for the TEAD4 gene that have not been previously identified, we investigated the epigenetic landscape of the TEAD4 gene as well as the flanking chromosomal regions using ENCODE datasets (Fig. 5A).

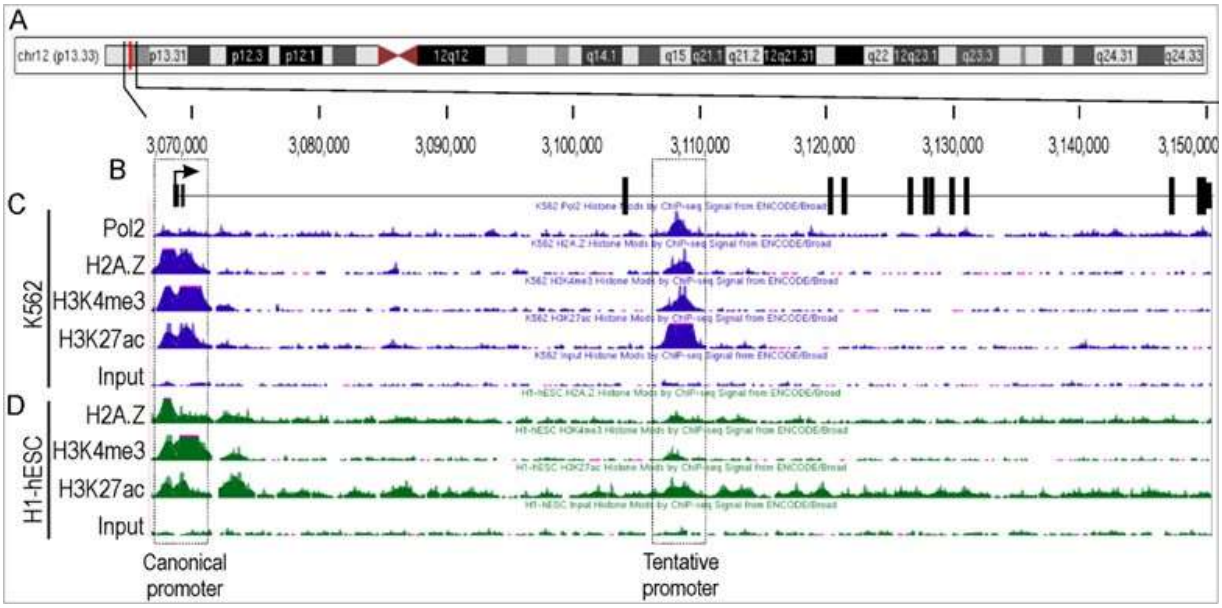

Figure 5. Epigenetic landscape of human TEAD4 encoding chromosomal region

The epigenetic context of the TEAD4 gene locus in immortalized cell lines can be detailed as follows: (a) On human chromosome 12, the exact position of the TEAD4 gene is marked by a vertical red line. (b) The structural organization of the TEAD4 gene, including its exons and introns, is depicted. Exons are represented by thick vertical lines, while the thinner horizontal lines signify the intronic regions. The direction of gene transcription is denoted by arrows. Additionally, the epigenetic profiles, including histone modifications and RNA polymerase II (Pol2) ChIP-Seq data, are presented for two cell lines: K562 (c), a human leukemia cell line, and H1-hESC (d), a human embryonic stem cell line.

Initially, the focus was on epigenetic histone signals, which are widely recognized as capable of characterizing transcriptionally active promoter regions. For example, the tri-methylation of histone H3 at lysine 4 (H3K4m3) and lysine 27 (H3K27ac), as well as the deposition of the histone 2A Z-isoform (H2A.Z), are well-established indicators of the initiation of transcription.

Furthermore, the presence of a transcriptionally active promoter is strongly indicated when the RNA polymerase II (Pol2) signal-peak overlaps with these signals. In the intergenic region of the TEAD4 gene, we observed the overlap of all these signals (Fig. 5C), suggesting the existence of a new hypothetical promoter embedded in the 3rd intron. Additionally, the ENCODE database provides ChIP-Seq data for multiple transcription factors. This data reveals a significant enrichment of transcription initiation signals within specific intronic regions that coincide with histone modifications indicating active promoters (see Figure 7). The analysis was conducted on two well-known cell lines: K562, which is derived from human myelogenous leukemia, and H1-hESC, a totipotent human embryonic cell line. The decision to concentrate on these cell lines is based on the ENCODE database's discovery that the intronic region of the TEAD4 gene has a distinct epigenetic landscape that promotes transcriptional activity. The analysis identified two areas with overlapping epigenetic markers, indicating strong transcriptional activity. One is the recognized canonical TEAD4 promoter, and the other is located approximately 40 kb downstream in intron 3. The predicted promoter region mentioned earlier has not been investigated yet. ChIP-Seq data indicates that it is functional only in certain cell types. This implies that transcripts originating from this alternative TEAD4 promoter may have unique roles in specific cellular contexts.

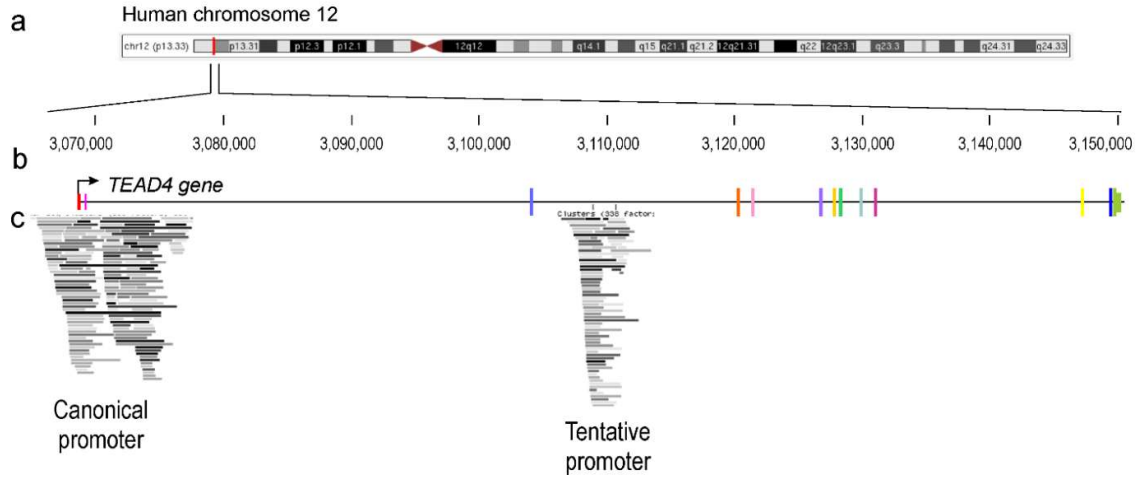

Figure 6. The ChIP-Seq data for transcription factors at the TEAD4 locus (a) The TEAD4 gene is positioned on the left arm of chromosome 12 in humans, marked by a vertical red line. (b) The structure of the TEAD4 gene, including its exons and introns, is depicted with exons highlighted by colored vertical lines and introns by thin horizontal lines. (c) The presence of ChIP-Seq data for certain transcription factors is indicated by gray horizontal lines, pinpointing their locations.

4.2. The presence of an in silico predicated TEAD4 promoter is confirmed by experimental results

To determine the transcriptional activity and pinpoint the transcription start site (TSS) of the putative promoter, a 5'RACE experiment was conducted using total RNA extracted from K562 cells. The cells were previously validated for transcriptional competence in silico investigations (see Figure 6). The obtained fragment was then PCR amplified, integrated into a suitable cloning vector, and subjected to Sanger sequencing to precisely identify the TSS. After aligning the nucleotide sequence of the cloned fragments with the human genome, we identified a novel transcription start site (TSS) within intron 3 (see Figure 8a, lane 1). It is noteworthy that this TSS coincided with the genomic region anticipated by epigenetic signals (see Figure 6). To develop transcript-specific primer pairs, we used this information to design a forward PCR primer that bound to the alternative exon and a reverse primer positioned in the 3'UTR. After amplification, the segment was cloned and sequenced using Sanger sequencing. The data analysis indicates that the exon identified by the new promoter is non-coding. However, the entire transcript produces a truncated form of TEAD4. Notably, the C-terminal section is identical.

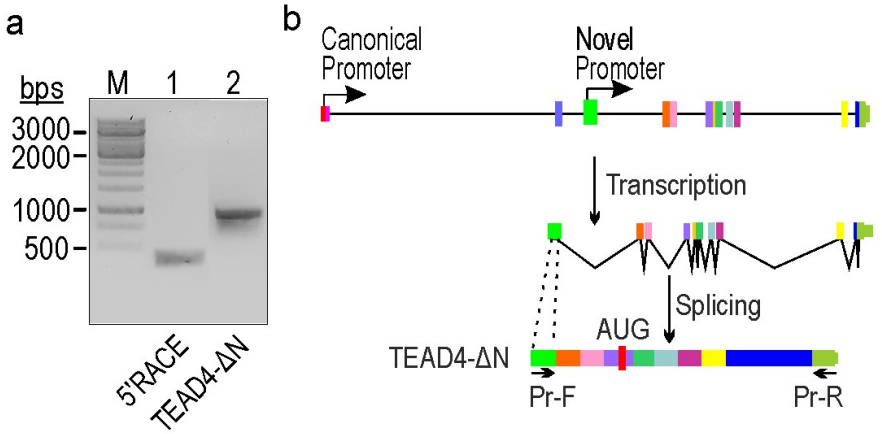

Figure 7. The discovery of a new TEAD4 isoform, which is produced from an alternative promoter located within an intron. (a) This part of the figure illustrates the 5'RACE (Rapid Amplification of cDNA Ends) analysis conducted on the TEAD4 gene. (b) Here, the exon/intron layout of the TEAD4 gene is depicted, along with the transcriptional and splicing processes that lead to the creation of the TEAD4-ΔN isoform. The positions of the PCR primers, labeled as Pr-F and Pr-R, used in the amplification of this novel transcript, are also indicated in this section.

The nucleotide sequence of the alternative mRNA initiated from the 3rd intronic region was determined by Sanger sequencing, as shown in Figure 9.

>TEAD4-ΔN

CCAGCCCTCCCCTTGAGCACCTCTTACCCTCCTGGGCCGGCTCCCGAGCCCGGGGTGCTTGCCTTCTCCTGCT
CACGGCCGCTTTCATTTCTGCCCTGTCACTGTGTGACCCTCCCCCTGGCACAGCCAACGCTGGCCACCCTGAC
CTCCTTTGGCCAGGCTCACAGTCGGCCTAGCCTAGCGTGCAGGTCGGAACGAGCTGATTGCCCGCTACATCAA
GCTCCGGACAGGGAAGACCCGCACCAGGAAGCAGGTCTCCAGCCACATCCAGGTGCTGGCTCGTCGCAAAGCT
CGCGAGATCCAGGCCAAGCTAAAGGACCAGGCAGCTAAGGACAAGGCCCTGCAGAGC**ATGGCTGCCATGTCGT**
CTGCACAGATCATCTCCGCCACGGCCTTCCACAGTAGCATGGCCCTCGCCCGGGGCCCCGGCCGCCCAGCAGT
CTCAGGGTTTTGGCAAGGAGCTTTGCCAGGCCAAGCCGGAACGTCCCATGATGTGAAGCCTTTCTCTCAGCAA
ACCTATGCTGTCCAGCCTCCGCTGCCTCTGCCAGGGTTTGAGTCTCCTGCAGGGCCCGCCCCATCGCCCTCTG
CGCCCCCGGCACCCCCATGGCAGGGCCGCAGCGTGGCCAGCTCCAAGCTCTGGATGTTGGAGTTCTCTGCCTT
CCTGGAGCAGCAGCAGGACCCGGACACGTACAACAAGCACCTGTTCGTGCACATTGGCCAGTCCAGCCCAAGC
TACAGCGACCCCTACCTCGAAGCCGTGGACATCCGCCAAATCTATGACAAATTCCCGGAGAAAAAGGGTGGAC
TCAAGGATCTCTTCGAACGGGGACCCTCCAATGCCTTTTTTCTTGTGAAGTTCTGGGCAGACCTCAACACCAA
CATCGAGGATGAAGGCAGCTCCTTCTATGGGGTCTCCAGCCAGTATGAGAGCCCCGAGAACATGATCATCACC
TGCTCCACGAAGGTCTGCTCTTTCGGCAAGCAGGTGGTGGAGAAAGTTGAGACAGAGTATGCTCGCTATGAGA
ATGGACACTACTCTTACCGCATCCACCGGTCCCCGCTCTGTGAGTACATGATCAACTTCATCCACAAGCTCAA
GCACCTCCCTGAGAAGTACATGATGAACAGCGTGCTGGAGAACTTCACCATCCTGCAGGTGGTCACCAACAGA
GACACACAGGAGACCTTGCTGTGCATTGCCTATGTCTTTGAGGTGTCAGCCAGTGAGCACGGGGCTCAGCACC
ACATCTACAGGCTGGTGAAAGAATGAGAGACTCGGGGAGCAGGGAGGGGGGAAG

Figure 8. The nucleotide sequence of newly discovered cDNA encoding new TEAD4 isoform. Alternating exons highlighted in yellow and gray. A novel (non-coding) exon within this sequence is underscored in red, and the open reading frame (ORF) is emphasized in bold.

The nucleotide sequence analysis of the known TEAD4 cDNA and the new cDNA (see Figure 9) revealed that the 5' region is missing and only the 3' region is present, which is identical (Figure 10). Actually, there is some extra sequences at the 5’ end, however it is a non-coding exon from the 3rd intron.

Figure 9. Pairwise comparison of mRNAs for TEAD4 isoforms. The alignment showcases the nucleotide sequences of the complete coding transcript for TEAD4 alongside the transcript encoding the recently discovered TEAD4-ΔN isoform. Identical nucleotides in the alignment are highlighted in red.

4.3. The Novel TEAD4 Isoform Encodes a DNA-Binding Domain-Less Protein

To identify the newly discovered transcript from the anticipated intronic region, we strategically designed and utilized a specialized set of PCR primers. This approach successfully amplified the mRNA in question, which originates from the third intron and extends through all subsequent canonical exons. In addition to this novel transcript, we also detected the well-known full-length

variant of the gene. As shown in Figure 9, this recently discovered transcript is significantly shorter than the full-length TEAD4 variant, TEAD4-FL.

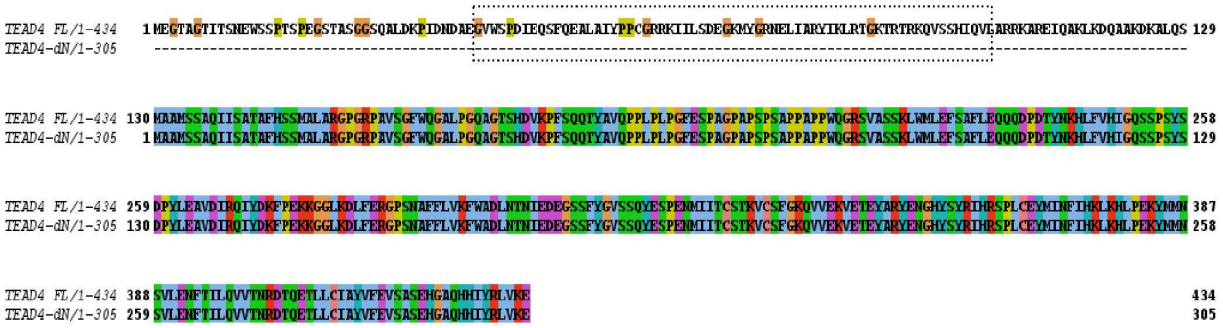

Figure 10. Comparison of the protein sequences of different TEAD4 isoforms. The figure includes the amino acid sequence of the full-length TEAD4 protein, labeled as TEAD4-FL. It also presents the sequence of the TEAD4-ΔN, an isoform characterized by the deletion of the N-terminal DNA binding domain. The DNA binding domain, typically present in the full-length version, is indicated by a dotted box in the figure, highlighting its absence in the TEAD4-ΔN isoform.

4.4. TEAD-ΔN isoform is excluded from the nucleus

Various regulatory mechanisms control the subcellular distribution of proteins. For instance, inhibitory proteins mask the nuclear localization signal (NLS) of NF-κB, preventing its constant movement into the nucleus (32). However, proteins without an NLS can still function effectively through mechanisms that enable NLS-independent nuclear localization (33). The deletion of the DNA-binding domain in the TEAD-ΔN isoform raises questions about its cellular and molecular functions. The NLS prediction tools were unable to identify the sequence in this truncated version of TEAD4. In order to investigate the cellular distribution of the isoform, we conducted experiments to determine its location. Our hypothesis was that understanding where the isoform localizes could provide insight into its potential role. To achieve this, we inserted both isoforms into plasmids that code for fluorescent proteins. The plasmid encoding GFP was used to insert the full-length TEAD4 (TEAD4-FL), while the plasmid coding for RFP was used to insert TEAD4-ΔN. These recombinant plasmid constructs were then transiently co-transfected into eukaryotic cells. The observations revealed that the full-length TEAD4 isoform predominantly localized in the nucleus, while the truncated TEAD4-ΔN was primarily observed in the cytoplasm (Figure 10). The presence of the TEAD4 isoform lacking the DNA binding domain in the cytoplasm may have implications for signal transmission in the Hippo pathway. We will discuss this topic later.

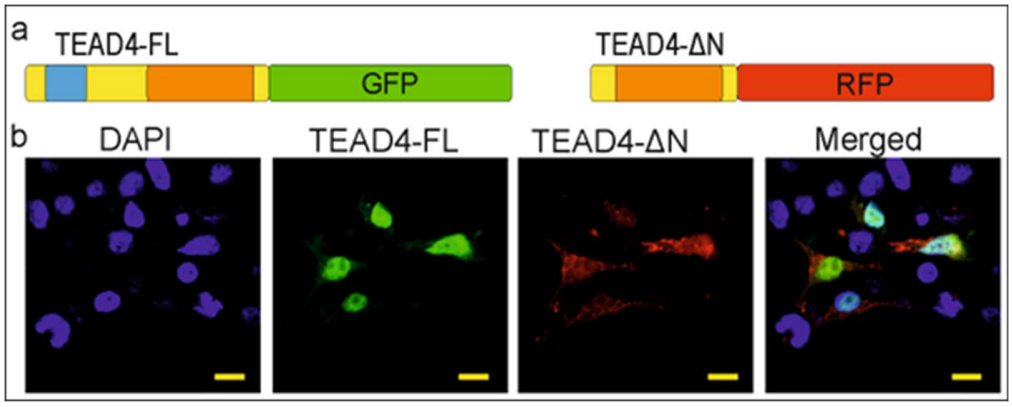

Figure 11. Subcellular Localization of TEAD4 Isoforms. (a) The diagram represents fusion constructs of the TEAD4 isoforms. The DNA-binding domains are marked with a light blue box, while the YAP-binding domains are shown in orange boxes. Both the green fluorescent protein (GFP) and red fluorescent protein (RFP) are utilized as fusion tags in these constructs. For the experiment, HEK293 cells were co-transfected with recombinant plasmids that express either the full-length TEAD4 (TEAD4-FL) or the truncated TEAD4 (TEAD4-ΔN) isoforms. Nuclear staining of these cells was conducted using DAPI, a nuclear-specific dye. The resultant images were captured at a high magnification of 800x, and include a scale bar of 10 μm for size reference.

4.5. TEAD4-ΔN expression is cell type-specific

This study investigated the expression of TEAD4 isoforms in various normal human tissues using isoform-specific endpoint PCR. Both full-length and truncated TEAD4 isoforms were assessed in the tissue samples. The results showed that the full-length TEAD4 isoform was present in all tissues analyzed, as depicted in Figure 11. However, the expression of the TEAD4-ΔN variant seems to be limited to specific cell types, suggesting a potentially more intricate role for this newly identified isoform.

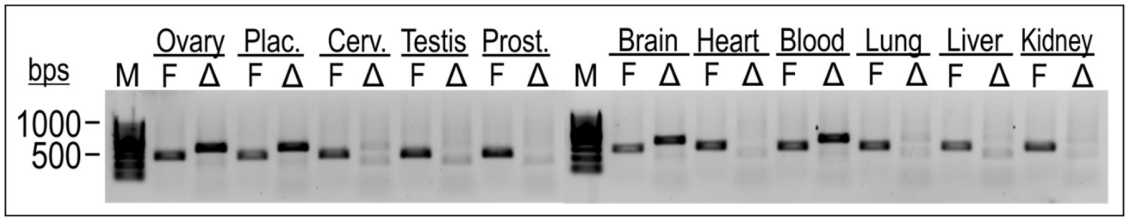

Figure 12. Isoform-specific end-point PCR analyzing the expression patterns of TEAD4 isoforms across various tissues. 'F' represents the full-length TEAD4 isoform (TEAD4-FL), and 'Δ' symbolizes the truncated variant, TEAD4-ΔN.

Figure 12 shows the results of Western blot analysis conducted to define the protein expression profiles of the TEAD4 isoforms in different stable cell lines. The longer TEAD4 isoform was found in all the cell lines examined, consistent with the results from the PCR studies. In contrast, the shorter TEAD4 isoform was detected exclusively in certain cell lines and exhibited notably lower expression levels compared to the longer variant. The RNA and protein data obtained from these experiments indicate clear differences in the expression levels and distribution patterns of the truncated TEAD4 isoform.

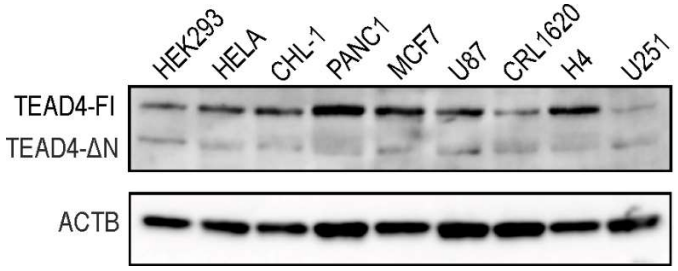

Figure 13. Protein Expression Profiles of TEAD4 in Different Cell Lines

4.6. In vitro analysis of the alternative TEAD4 promoter

Transcriptional control involves the collective interaction of transcription factors (TFs) at promoter and enhancer regions. These interactions facilitate the formation of preinitiation complexes, leading to effective transcription by the RNA polymerase II (Pol2) enzyme. An exploration of the TEAD4-ΔN promoter for TF binding sites revealed several potential sites, including a consensus motif for TEAD4 itself. This finding suggests a possible self-regulatory mechanism for TEAD4. To confirm the binding of TEAD4 to the predicted consensus motif within the TEAD4-ΔN promoter, we performed electrophoretic mobility shift assays (EMSAs) (Figure 14a). As high-quality TEAD4 antibodies for chromatin immunoprecipitation (ChIP) assays were not commercially available, we used a competitive EMSA method to validate the binding of TEAD4 to this newly identified promoter region. The nucleotide sequence of the cis-element predicted for TEAD4 binding closely resembles the known consensus sequence for TEAD4, as shown in Figure 14b. The core six nucleotides of this binding site are identical, with only one variation in a flanking nucleotide. Figure 14 highlights the differences in binding efficiency between the consensus sequence and the actual TEAD4-ΔN promoter-related motifs in lanes 2 and 7. Consequently, the EMSA results showed two TEAD4-specific bands (marked with asterisks). Band B was found to be uniquely associated with TEAD4, as demonstrated by its absence with the mutated promoter

element (Figure 14, lane 5) and its elimination upon competition with a TEAD4 consensus oligonucleotide (Figure 14, lane 4). Although TEAD4 may contribute to the formation of band A, it is clear that other interacting transcription factors also play a role in the formation of this DNA-protein complex.

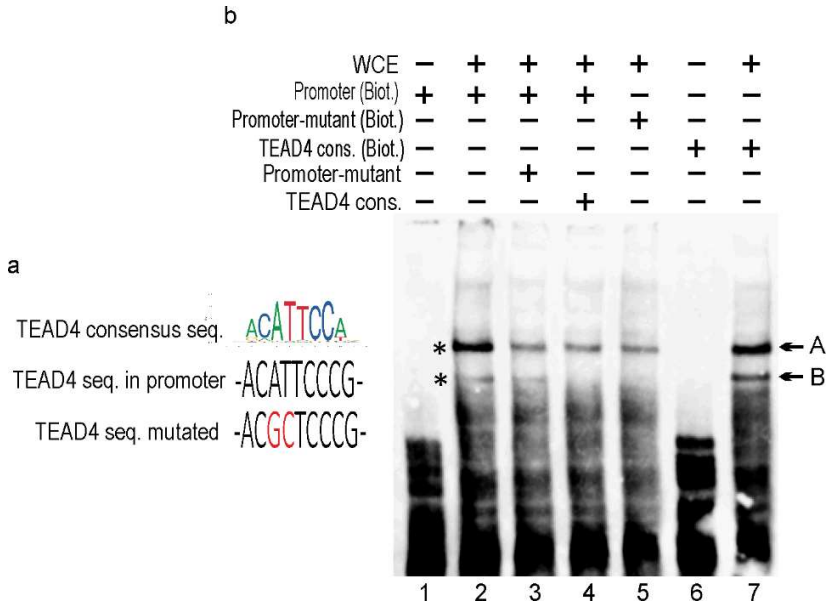

Figure 14. Analysis of in vitro DNA-Protein Interactions Using EMSA. The upper panel highlights the TEAD4 consensus motif, displayed in color, while the middle panel presents the TEAD4 motif identified in the TEAD4-ΔN promoter. The red label points out the core nucleotides that are mutated in the TEAD4 binding site. The lower panel specifies the components used in the binding reaction, indicated with +/- symbols. WCE refers to Whole Cell Extract; Prom-(B) represents the biotinylated promoter element containing a TEAD4 binding site; Prom Mut-(B) denotes the biotinylated promoter element with a mutated TEAD4 binding site; TEAD4-(B) is the biotinylated TEAD4 consensus sequence; and Prom-Mut and TEAD cons are the unbiotinylated double-stranded competitor oligonucleotides.

4.7. Functional characterization of TEAD4 promoter(s) in transient transfection studies

To deepen our understanding of how TEAD4-ΔN is regulated at the transcriptional level and to test the relevance of our in vitro DNA-protein interaction findings, we PCR-amplified the promoter region upstream of the transcription start site (TSS) and linked it to a luciferase reporter gene. We chose a 1.3-kilobase segment of the intron, as highlighted in the Ensembl Regulatory Build (92)—a detailed repository of epigenetic markers and transcription factors—because it succinctly outlines potential regulatory areas for TEAD4-ΔN promoter region (Figure 15).

AGCTGAATGGCCTTGCTCCTCCACTGCCTGGCCCTGGCTCCAGGTGGGTCAGGCCCTGGGCAGGGCCCTG
GAGGCTGTACTGCAGGCTCTGACAACCGCCCAGCCCCCACAGCACCTGTCCTGCATGAGAATAGGTTCTG
CTGGGATCCAGACACCCGCTCACCCCGTCTCTTCTTACCAGTCCCGGGGGCCGAGCCAAGGCAGGACTGC
GTGGGGACTGATGGAGGGCTCTCAGCAGGCAGTGCTGACCAGCCCGGCTGGGAGCTATAGATAGCTCTGA
GCTCACCCCCAGTGTGTGGGATGGGCCCTCTGGGAGGCGGAATGGGAGGCCGAGCTGAGAGCTGACTCAG
ACCTCAGCTCACCGTGGCTGCTCCCCTCCTGTCTTCTCATCTTCCCTTGGGGCATCTGCGCTTCCCATCC
TCTGTGTACCCCAGCCCCATTCTGAGCCCCCAGCTGCTTCTAGCATCCCCAGAGCTCTGGTCTTTTCTCC
CCCCTCACTTCCCTGCATCCAAAGGCCACCAGCCCCTTTCCAAGTGGGCCGGCCCGGTGGGGTATGGGGT
GGGGCTAAGAGGATGATTCCCGACCTGGGGACCCGGCCTTAGCTGTTTGAGGACAGGGGTTAGGCCTGCT
CCGAGCTCCGCCCACGGAACATCCAGCATAGGACACGCTGACCAAGGCCAGGCAGACATGCAGATGACAT
GCAAAGCAGCACGGCAGATTAACACCTGCTATTTCTGAGCTGAGTCTCCCACGCTTGTTGGCTCCAGCCC
TCCCCTTGAGCACCTCTTACCCTCCTGGGCCGGCTCCCGAGCCCGGGGTGCTTGCCTTCTCCTGCTCACG
GCCGCTTTCATTTCTGCCCTGTCACTGTGTGACCCTCCCCCTGGCACAGCCAACGCTGGCCACCCTGACC
TCCTTTGGCCAGGCTCACAGTCGGCCTAGCCTAGCGTGCAGGTGGTGGGATTCGAACAACTTCTCGCCCC
CTTCTGCCTGCTCCCCGCCTTCCCCGCTCCTCACACTCAGTGCTGGCCGGGTGGGCTCGTGTCCCGCCTC
CTGCTCTCTGGAGGGCTGTGGCTGGCTCTGGGGTTCCCTCTGCATTCATCCCTTTCTGCCTCCTGTGCTT
CTCACCTTCCTGAGGTTGCTCTTGACTTTCCCGAGTCTTCCTGCCTCTTTTCCCTGCCTCTCTCTCTGCT
TCTCCCCTCCTGTGTCCCAGGTGGGCCATCCTATTAGCAGCCCGTCAGTTCTCATTAAGTGACCACCCCA
CACTGGGCAGGCCGGGCTGAGGCCGTGTGGTCTCTGCTCTCCACAACTTCATGGTCTAATGAGAGGGGCA
GGAAAAACTTCTCTGGACAGTTAGCCACCCAAGCAGACACTGGGGTTGTCCACAG

Figure 15. The nucleotide sequence of the TEAD4-ΔN promoter. The green highlighted regions are very conserved among mammals.

The region depicted in Figure 15 is evolutionarily conserved in mammals, suggesting a significant role in transcriptional regulation. To analyze its function in a eukaryotic context, we constructed recombinant luciferase reporter plasmids containing the promoters for both canonical TEAD4 and TEAD4-ΔN and transfected them into HEK293 cells (Figure 15). In our assays, the newly identified promoter exhibited significant luciferase activity, although less intense than that of the canonical promoter. This suggests that the isoform with the DNA-binding domain may have a more prominent role in cellular function, while the TEAD4-ΔN isoform could be important in specific situations and cell types. In vitro experiments have shown that the TEAD4 transcription

factor can bind to the TEAD4 cis-element located in the novel intronic promoter, as demonstrated in Figure 16.

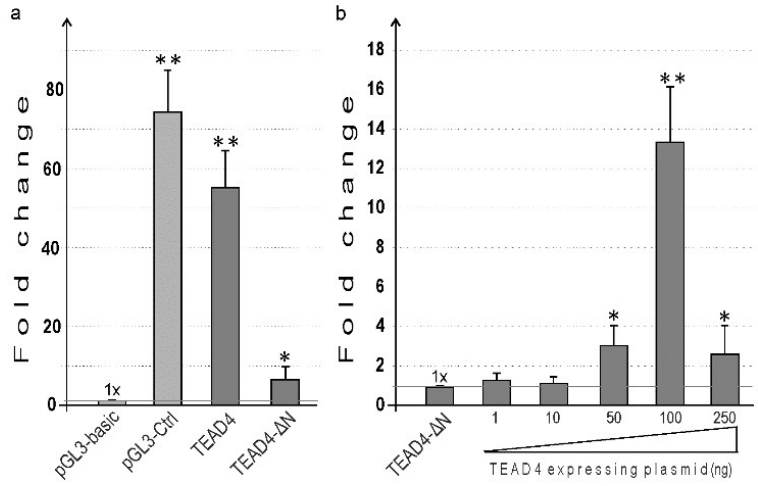

Figure 16. The functional analysis of TEAD4 promoters through transient expression assays. Both recombinant and control plasmids were transiently transfected into cells, with luciferase activity measurements taken 48 hours post-transfection. (a) This part of the study successfully identifies the functionality of the TEAD4-ΔN promoter. The pGL3-basic plasmid, lacking any promoter, served as a control. The luciferase activity in cells transfected with the pGL3-basic plasmid was normalized to protein content and set as the baseline (1-fold). pGL3-Ctrl, the control plasmid for transfection, is driven by the SV40 viral promoter and enhancer. Two plasmids were utilized for TEAD4: one containing a 1 kb-long canonical promoter (TEAD4) and the other a 1.3 kb-long intronic region inserted minigene construct (TEAD4-ΔN). (b) This segment investigates the impact of overexpressing the TEAD4 transcription factor on the TEAD4-ΔN promoter-driven luciferase minigene. The luciferase activity in cells transfected with the TEAD4-ΔN plasmid was normalized to protein content as the standard (1-fold), and the luciferase activities of other samples were compared against this value. Various concentrations of TEAD4 overexpressing plasmids were co-transfected with the TEAD4-ΔN reporter construct. The presented data show the mean ± standard error of the mean (SEM). Statistical significance is indicated as $*p \leq 0.05$, $**p \leq 0.01$.

To explore the effects of TEAD4's interaction with this promoter region, we co-transfected a plasmid overexpressing TEAD4 along with a reporter gene construct containing the 1.3 kb TEAD4-ΔN promoter. We observed that as the amount of the TEAD4-overexpressing plasmid increased, there was a corresponding rise in promoter activity, indicated by heightened luciferase activity. This suggests that TEAD4 acts as a positive regulator of its truncated isoform's expression (Figure 16b). However, it's important to note that excessively high levels of TEAD4 can potentially inhibit promoter activity. The data presented from both in vitro and in vivo studies collectively

demonstrate that TEAD4 modulates the expression of its own isoform by interacting with its novel intronic promoter region.

4.8. TEAD4-ΔN expression in human placenta is regulated by DNA methylation

TEAD4 plays a critical role in ensuring the survival of human embryos post-implantation by controlling the self-renewal and development of trophoblast progenitors in the placental primordium (93) .Studies indicate that in mice, the absence of TEAD4 in trophoblast stem/progenitor cells (TSPCs) post-implantation leads to a reduced self-renewal capacity, resulting in embryonic fatality before embryonic day 9.0. This developmental stage is equivalent to the first trimester of pregnancy in humans (93). Thus, understanding the role of TEAD4-ΔN expression during this critical developmental stage is of significant interest. As a preliminary step, we examined the expression patterns of TEAD4 isoforms in human placenta and umbilical cord tissue samples, as shown in Figure 17.

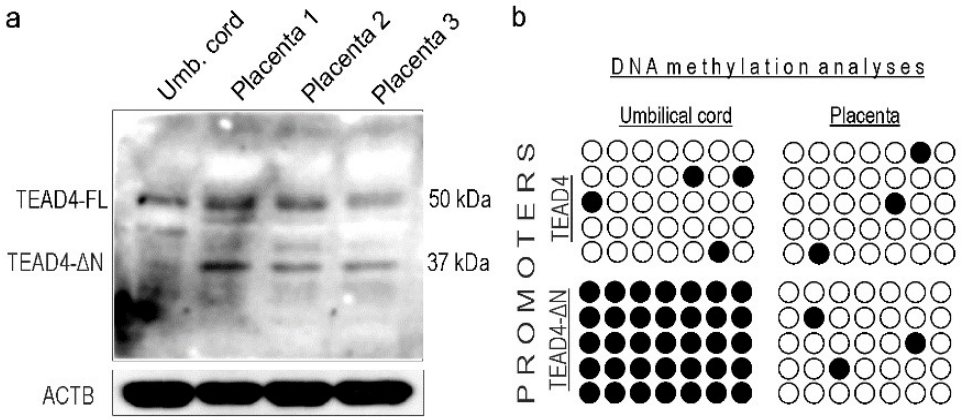

Figure 17. Combined analysis of protein expression and DNA methylation in human placental samples. (a) The expression levels of TEAD4 isoforms were examined through western blotting. ACTB (β-actin) served as the loading control for these assays. (b) The bisulfite sequencing approach was utilized to analyze the methylation status of TEAD4 promoters. In this analysis, open circles indicate unmethylated CpG sites, whereas closed circles represent methylated CpG sites.

Our analysis revealed distinct expression profiles for the two TEAD4 isoforms in these human samples. Notably, TEAD4-ΔN was absent in the umbilical cord samples but was detected in the placental lysates. This differential expression suggests a unique role for the TEAD4-ΔN isoform in placental development. Epigenetic modifications, such as DNA methylation and various histone

alterations, create a chromatin environment conducive to efficient transcription. Building on this, we delved into the potential epigenetic mechanisms that might govern the selective expression of TEAD4 isoforms. We performed DNA methylation analysis, including bisulfite sequencing, on genomic DNA extracted from umbilical cord and placenta samples. This epigenetic exploration targeted the two TEAD4 promoters – the canonical and the alternative – to discern their methylation status, which could elucidate the differential protein expression observed. The results from our bisulfite sequencing aligned with the protein expression patterns we noted. In both umbilical cord and placenta samples, the canonical TEAD4 promoter exhibited no methylation, correlating with the consistent expression of the TEAD4 protein in these tissues. Conversely, the promoter for the truncated TEAD4-ΔN isoform showed substantial methylation in umbilical cord samples, leading to transcriptional suppression and, consequently, the absence of TEAD4-ΔN isoform expression in these samples. This finding helps clarify the protein expression patterns we detected via Western blot analysis.

5. DISCUSSION

The discovery of a novel TEAD4 isoform, initiated from an alternative intronic promoter region, marks a significant advancement in our understanding of gene regulation. This isoform, TEAD4-ΔN, presents as an N-terminus truncated variant of the extensively studied TEAD4 transcription factor. Intriguingly, the TEAD4-ΔN transcript lacks the conventional DNA-binding domain (TEA/ATTS), indicating a potential divergence from the canonical transcriptional regulatory roles typically associated with TEAD4. This hypothesis is further substantiated by observations of the TEAD4-ΔN:RFP chimera protein, which, devoid of a nuclear localization signal (NLS), predominantly localizes in the cytoplasm of transfected cells, suggesting a non-nuclear function.

Prior research has documented a shorter TEAD4 isoform attributed to alternative splicing [30]. Our efforts to detect the mRNA from this splicing event across various cell types were unsuccessful, highlighting its rarity or specific condition-dependent expression. The literature associates the shorter isoform's generation with tumor-related exon skipping, considered an infrequent occurrence. However, our findings propose that alternative promoter usage, potentially a more common event, also leads to the production of this isoform, challenging the notion that its emergence is strictly linked to tumorigenesis.

The function of TEAD4-ΔN at the molecular and cellular levels remains largely speculative. Notably, the transcript retains the full-length YAP-binding domain, raising possibilities of its interaction with YAP1 protein in the cytoplasm and subsequent influence on cellular signaling mechanisms. The phosphorylation and nuclear translocation of YAP1, a key aspect of the Hippo signaling pathway, are critical processes in regulating cell proliferation and organ size. Therefore, understanding how TEAD4-ΔN impacts the Hippo pathway, particularly its regulation and the differentiation processes crucial for embryo implantation and placental formation, could provide insights into the intricate mechanisms governing these vital developmental stages. Our study demonstrates that TEAD4-ΔN mRNA expression is closely linked to the DNA methylation status of its intronic promoter. A lack of methylation in this region could foster a more open chromatin configuration, enhancing nucleosome de-condensation and facilitating the binding of various transcription factors, including those possessing DNA-binding domains. Such an environment could augment the gene regulatory roles of TEAD4, as it can form heterodimers with other TEAD family proteins and SMAD transcription factors, further influencing gene expression (94).

The role of DNA methylation in epigenetic regulation, particularly during the critical pre- and post-implantation periods of embryonic development, is well established (95)DNMT3B, a key enzyme in de novo DNA methylation, is essential in modulating placental development and function (96). The methylation-dependent regulation of TEAD4-ΔN thus opens new avenues of inquiry into the functional implications of this isoform, particularly in the context of placental development and embryonic growth. TEAD4, in its traditional form lacking a DNA-binding domain, is known to disrupt the Hippo-YAP signaling pathway, affecting cell proliferation, migration, and organ growth (97). However, the specific molecular function of TEAD4-ΔN, including its interaction with other proteins and its role in various signaling pathways, remains to be fully elucidated. Uncovering these mechanisms could have significant implications for therapeutic interventions and diagnostics. For instance, manipulating TEAD4-ΔN expression could offer new strategies in cancer therapy, potentially disrupting malignancy-associated signaling pathways. Furthermore, TEAD4-ΔN could serve as a biomarker for certain cancers, providing diagnostic and prognostic value.

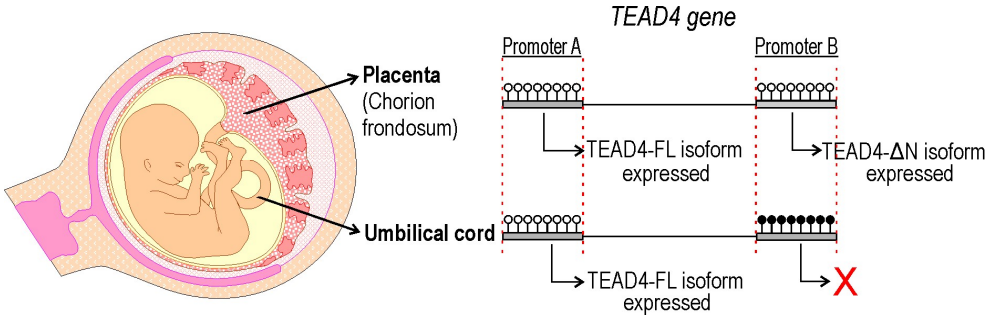

Figure 18. DNA Methylation and Its Impact on TEAD4 Isoform Expression. This figure illustrates the role of DNA methylation in regulating the expression of different TEAD4 isoforms. Promoter A, which drives the expression of the full-length TEAD4 (TEAD4-FL), remains unmethylated across various tissues, ensuring the ubiquitous presence of this isoform. In contrast, Promoter B, responsible for the expression of the truncated isoform (TEAD4-ΔN), exhibits tissue-specific methylation patterns, leading to a differential presence of this isoform in various tissues. The graphic representation includes CpG dinucleotides depicted as lollipops; open lollipops represent unmethylated CpGs, whereas those filled in black indicate methylated CpGs.

6. SUMMARY

The discovery of a novel TEAD4 isoform, TEAD4-ΔN, originating from an alternative intronic promoter, suggests a new understanding of gene regulation. This truncated variant lacks the conventional DNA-binding domain, indicating a potential shift from TEAD4's typical transcriptional roles. The TEAD4-ΔN isoform is mostly found in the cytoplasm, suggesting a non-nuclear function. It is associated with alternative promoter usage rather than strictly with tumorigenesis. The isoform retains the YAP-binding domain, hinting at interactions with YAP1 in the cytoplasm and potential effects on cellular signaling. TEAD4-ΔN's expression is linked to DNA methylation status, impacting gene regulation and potentially influencing placental development and embryonic growth. The identification and preliminary characterization of TEAD4-ΔN as described in Figure 18, represents a pivotal step forward in the field of gene regulation and epigenetics. Understanding the unique functions and regulatory mechanisms of this isoform could provide valuable insights into cellular processes during development and disease, potentially leading to novel therapeutic strategies and diagnostic tools. As we continue to explore the diverse roles of TEAD4 variants, their impact on cellular signaling, development, and disease remains an intriguing and vital area of research.

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8. LIST OF PUBLICATIONS

Peer-Reviewed Papers:

The paper is directly associated with the PhD thesis.

Rashidiani S, Mamo G, Farkas B, Szabadi A, Farkas B, Uszkai V, Császár A, Brandt B, Kovács K, Pap M, Rauch TA. Integrative epigenetic and molecular analysis reveals a novel promoter for a new isoform of the transcription factor TEAD4. Int J Mol Sci. 2024 Feb 13;25(4):2223. doi: 10.3390/ijms25042223.PMID:38396900.

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Other publications:

1. Mamo G, Rashidiani S, Farkas B, Szabadi A, Farkas B, Brandt B, Pap M, Rauch TA. Unveiling the Role of Exosomes in the Pathophysiology of Sepsis: Insights into Organ Dysfunction and Potential Biomarkers. Int J Mol Sci. Accepted for publication on April 30, 2024

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2. Ahmadi H, Aghebati-Malek, Rashidiani S, Csabai T, Basil Nnaemeka O; Szekeres-Bartho J. Long-Term Effects of ART on the Health of the Offspring. Int. J. Mol. Sci. 2023, 24(17), 13564.

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Conferences

1. Rashidiani S, Pap M & Rauch TA Epigenetic profiling of the human chromosome 12 (p13.33) region reveals a novel promoter of the TEAD4 transcription factor. Hungarian molecular life sciences, 24-26 March, 2023, Eger, Hungary.

2. Rashidiani S, Pap M & Rauch TA. DNA methylation governed expression of the alternative TEAD4 promoter. Annual Meeting of the Hungarian Biochemical Society 2022. 25-27 August 2022. Pécs, Hungary.

3. Rashidiani S, Pap M & Rauch TA, Epigenetic profiling of the human chromosome 12 (p13.33) region reveals a novel promoter of the TEAD4 transcription factor. 29th International Student Congress Of (bio)Medical Sciences. 8-10 June 2022, Netherlands.

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10. APPENDIX

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Article

Integrative Epigenetic and Molecular Analysis Reveals a Novel Promoter for a New Isoform of the Transcription Factor TEAD4

Shima Rashidiani [1], Gizaw Mamo [1], Benjámin Farkas [1], András Szabadi [1,2], Bálint Farkas [3,4], Veronika Uszkai [3], András Császár [3], Barbara Brandt [5], Kálmán Kovács [3,4], Marianna Pap [5,†] and Tibor A. Rauch [1,*,†]

[1] Institute of Biochemistry and Medical Chemistry, Medical School, University of Pécs, 7624 Pécs, Hungary; sh.rashidiani@gmail.com (S.R.); mamo_gizaw@yahoo.com (G.M.); benjamin.farkas@aok.pte.hu (B.F.); szabadi.andras@pte.hu (A.S.)
[2] Department of Dentistry, Oral and Maxillofacial Surgery, Medical School, University of Pécs, 7623 Pécs, Hungary
[3] Department of Obstetrics and Gynecology, Medical School, University of Pécs, 7624 Pécs, Hungary; farkas.balint@gmail.com (B.F.); uszkai.veronika@pte.hu (V.U.); csaszar.andras@pte.hu (A.C.)
[4] National Laboratory of Human Reproduction, University of Pécs, 7624 Pécs, Hungary
[5] Department of Medical Biology and Central Electron Microscope Laboratory, Medical School, University of Pécs, 7624 Pécs, Hungary; marianna.pap@aok.pte.hu (M.P.)
* Correspondence: tibor.rauch@aok.pte.hu
† These authors contributed equally to this work.

Abstract: TEAD4 is a transcription factor that plays a crucial role in the Hippo pathway by regulating the expression of genes related to proliferation and apoptosis. It is also involved in the maintenance and differentiation of the trophectoderm during pre- and post-implantation embryonic development. An alternative promoter for the TEAD4 gene was identified through epigenetic profile analysis, and a new transcript from the intronic region of TEAD4 was discovered using the 5′RACE method. The transcript of the novel promoter encodes a TEAD4 isoform (TEAD4-ΔN) that lacks the DNA-binding domain but retains the C-terminal protein–protein interaction domain. Gene expression studies, including end-point PCR and Western blotting, showed that full-length TEAD4 was present in all investigated tissues. However, TEAD4-ΔN was only detectable in certain cell types. The TEAD4-ΔN promoter is conserved throughout evolution and demonstrates transcriptional activity in transient-expression experiments. Our study reveals that TEAD4 interacts with the alternative promoter and increases the expression of the truncated isoform. DNA methylation plays a crucial function in the restricted expression of the TEAD4-ΔN isoform in specific tissues, including the umbilical cord and the placenta. The data presented indicate that the DNA-methylation status of the TEAD4-ΔN promoter plays a critical role in regulating organ size, cancer development, and placenta differentiation.

Keywords: TEAD4; Hippo/TEAD signaling; alternative promoter; transcriptional regulation; DNA methylation

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1. Introduction

Although organ-size coordination, tumor growth regulation, and trophectoderm differentiation are seemingly completely different cellular processes, they are linked by at least one common regulatory network, the TEAD/Hippo pathway. Originally identified in Drosophila in a screen for tissue growth regulators [1], it has since been shown that all essential components of the TEAD/Hippo regulatory cascade are present in mammals [2]. The TEAD/Hippo pathway comprises an intricate network of more than 30 core elements, including ligands, receptors, protein kinases, transcription factors, and transcriptional co-factors [3]. TEA-domain transcription factors (TEADs) belong to the transcription-enhancer factor (TEF) family, which has the TEA/ATTS DNA-binding domain and recognizes the

TGGAATGT consensus sequence in promoter regions [4]. The four TEAD proteins exhibit a high degree of homology, with a range of 61% to 73%, and possess a DNA-binding domain at the N-terminus and a YAP/TAZ-binding domain at the C-terminus. Nearly all tissues express at least one of the TEAD genes, and some express all four [5,6]. TEAD1 enhances the expression of genes specific to the heart and is believed to be critical for myocardial differentiation [7]. TEAD2 is involved in regulating gene expression during neural development [8]. The precise function of TEAD3 has yet to be elucidated. The regulatory function of TEAD4 was initially explored in the context of embryo implantation, unveiling its involvement in the differentiation of blastomeres into the trophectoderm [9,10]. In Tead4 mutants, there is a substantial decrease in Cdx2 expression in blastomeres, which then differentiate into inner-cell-mass cells, implying that Tead4 plays a critical role in the initiation of Cdx2 expression, a crucial gene in TE development [9,11]. The absence of TEAD4 results in lowered mitochondrial activity and heightened levels of oxygen-reactive species in pre-implantation mouse embryos [12]. Additionally, TEAD4 is detected in trophoblast stem-cell-like progenitor cells (TSPCs), and the loss of Tead4 in post-implantation mouse TSPCs impairs their self-renewal, resulting in embryonic lethality before 9.0 days of embryonic development, which corresponds to the first trimester of human pregnancy [13]. An accurate comprehension of the mechanism of action of the TEAD/Hippo cascade has been disclosed in the tumor context. Consequently, Hippo-signaling core kinases remain inactive at low cell densities, leading to unphosphorylated YAP1's translocation into the nucleus, where it interacts with TEAD4 [14]. The binding of TEAD4-YAP1 triggers cell proliferation through the activation of cell-division-promoting genes and anti-apoptotic genes. In contrast, when cells reach a point of contact-mediated inhibition (CMI), upstream modulators of the Hippo pathway, like E-cadherin, are activated, leading to the phosphorylation of YAP1. Phosphorylated YAP1 becomes degraded in the cytoplasm, eventually resulting in cell proliferation inhibition [15–17]. The TEAD/Hippo pathway undergoes downregulation during tumorigenesis, resulting in uncontrolled cell growth and tumor-cell metastasis. Recent genome-wide studies demonstrate that the association between DNA methylation and gene expression is more intricate than previously understood and is dependent on the specific genomic region involved (such as the promoter or intragenic region), which is often linked to the gene's epigenetic context [18]. DNA methylation is linked to reduced transcriptional activity in the promoter region, but highly methylated intragenic (i.e., intronic) areas are associated with elevated transcriptional rates [19,20]. Methylation, or its lack thereof, may determine the transcriptional activity of alternative promoters [21,22]. DNA methylation is not the only mechanism involved in the regulation of intragenic promoters, but it is associated with a specific epigenetic histone signal (e.g., H3K36me3). DNA and histone hypomethylation promote transcriptional activity of alternative promoters during tissue- and developmental-stage-specific gene expression [21,23,24]. A comprehensive epigenetic reprogramming takes place during early embryonic development, erasing the parental DNA methylation pattern and creating a new one with profound implications for the segregation of the trophectoderm and inner cell mass [10]. This critical change in the DNA methylation pattern has the potential to alter the patterns of gene and isoform expression of TEAD4.

Our study reveals that ChIP-Seq data analysis can predict an alternative promoter for the TEAD4 gene. Consequently, a previously unknown transcript is initiated from an intronic region of TEAD4 and is expressed only in certain tissues. The newly identified TEAD4 transcript encodes a truncated isoform (TEAD4-ΔN) that lacks the DNA-binding domain and is mainly localized in the cytoplasm. The alternative promoter of TEAD4 exhibits differential methylation between expressing and non-expressing cell types, suggesting a strict epigenetic control of isoform expression. Furthermore, TEAD4 interacts with the alternative promoter region, which results in the upregulation of TEAD4-ΔN expression. DNA-methylation-mediated epigenetic regulation of the novel promoter may be highly relevant in biological and pathological contexts (i.e., early mammalian development

and tumorigenesis) where DNA methylation plays a critical role in controlling genetic reprogramming and cancer-specific gene expression.

2. Results

2.1. Identification of a Novel Promoter for TEAD4

Major genetic/genomic databases, such as NCBI, ENCODE, and Ensembl, list only a single TEAD4 gene without any additional promoter(s) that produce alternative isoform(s). Typically, transcription factors with complex regulatory networks, like TEAD4, have numerous isoforms [21,25,26]. Our hypothesis was that the TEAD4 gene may also have alternative promoter(s) and that corresponding transcript(s) could play a role in fulfilling its intricate regulatory function. To identify novel and unexplored TEAD4 gene promoters, we analyzed the epigenetic landscape of the TEAD4 gene and its surrounding chromosomal regions using data from the ENCODE databases (Figure 1). Initially, we focused on the epigenetic histone signals that define the transcriptionally active promoter regions. For instance, there is strong evidence that the tri-methylation of histone H3 at lysine 4 (H3K4me3), the acetylation of histone H3 at lysine 27 (H3K27ac), and the deposition of the Z isoform of histone 2A (H2A.Z) are signals of transcription initiation. In addition, the presence of a transcriptionally active promoter is highly indicated if the RNA polymerase 2 (Pol2) signal peak aligns with these signals. Furthermore, the ENCODE database contains ChIP-Seq datasets for various transcription factors, which demonstrate that the intronic region is heavily enriched in certain regions that overlap with histone signals defining active promoters (Figure S1). Examination of these signals was conducted on two cell lines, K562, a human myelogenous leukemia cell line, and H1-hESC, a totipotent human embryonic cell line, both of which are well-characterized. The analysis focused on the H1-hESC and K562 cell lines due to the ENCODE database revealing that the intronic region of the TEAD4 gene has the most characteristic epigenetic milieu to support transcriptional competence. Two regions displayed overlapping epigenetic signals, implying substantial transcriptional potential. One region was the well-known canonical TEAD4 promoter, and the other was located ~40 kbs downstream of the canonical promoter in intron 3. The predicted promoter, which has not yet been explored, can only be functional in specific cell types, as evidenced by ChIP-Seq data. This implies that the corresponding transcript initiated by this alternative TEAD4 promoter might have a distinct role.

2.2. Identification of a Novel TEAD4-Isoform-Encoding Transcript

In order to determine the transcriptional activity and pinpoint the transcription start site (TSS) of the putative promoter, a 5'RACE experiment was conducted using total RNA extracted from K562 cells, which has been validated for transcriptional competence in previous in silico investigations (Figure 1). The obtained fragment was subsequently PCR-amplified (Figure 2a, lane 1), cloned into a suitable cloning vector, and subjected to Sanger sequencing to precisely identify the TSS. After aligning the nucleotide sequence of the cloned fragments with the human genome, we identified a novel TSS within intron 3. It is noteworthy that this TSS coincided with the genomic region anticipated by epigenetic signals (Figure 1). To generate transcript-specific primer pairs, we employed this knowledge and designed a forward PCR primer that binds to the alternative exon and a reverse primer positioned in the 3'UTR. After amplification (Figure 2a, lane 2), the fragment was cloned and sequenced using Sanger sequencing. Analysis of the data suggests that the exon identified by the new promoter is non-coding, whereas the entire transcript generates a truncated form of the TEAD4 protein (designated as TEAD4-DN). Interestingly, the C-terminal region of the truncated protein is identical to the full-length TEAD4 protein.

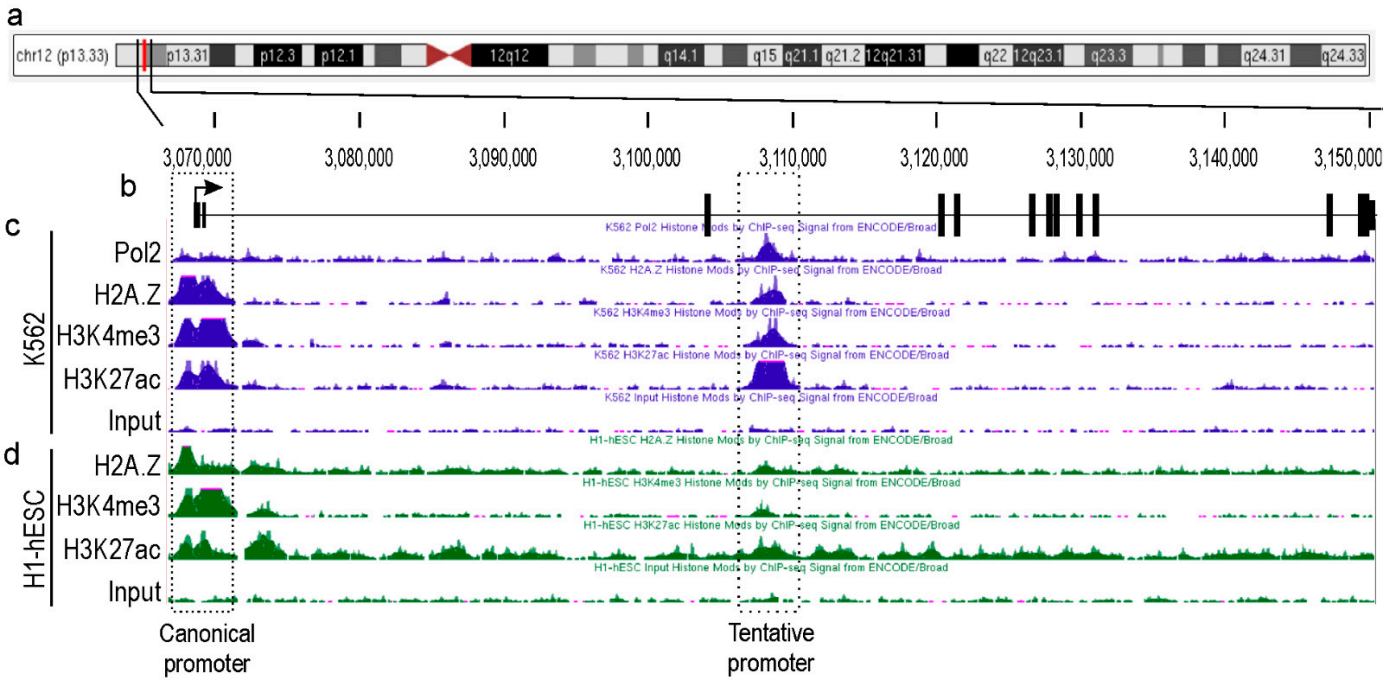

Figure 1. Epigenetic landscape of the TEAD4-gene-encoding locus in permanent cell lines. (**a**) Human chromosome 12. The vertical red line indicates the precise location of the TEAD4 gene on the chromosome. (**b**) Exon/intron structure of TEAD4 gene. The diagram indicates the exons by the thick vertical lines and the intronic regions by the thin horizontal lines. The direction of transcription is indicated by the arrow. The epigenetic histone and Pol2 ChIP-Seq profiles are shown for the K562 (**c**) and H1-hESC (**d**) cell lines.

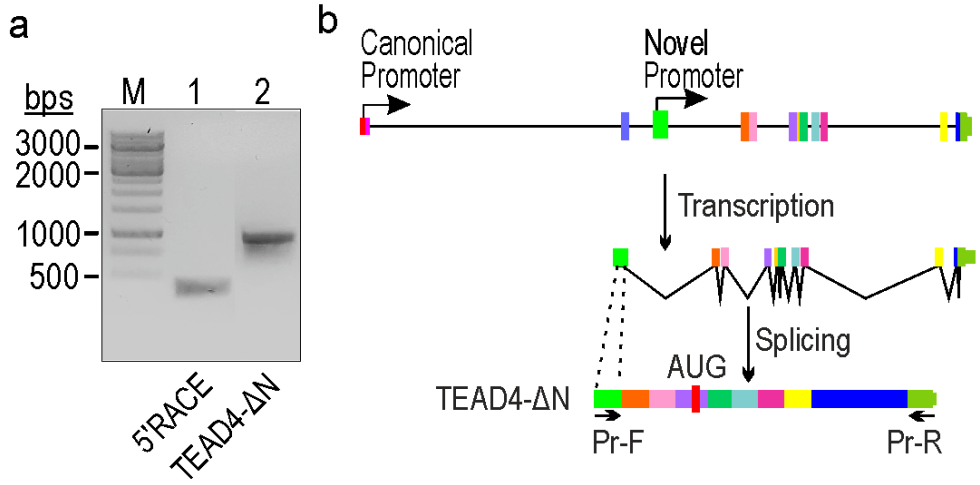

Figure 2. Identification of a novel TEAD4-isoform-encoding mRNA transcribed from an alternative intronic promoter. (**a**) 5′RACE analysis of the TEAD4 gene (lane 1) and the amplicon of the truncated form of the TEAD4 gene (lane 2). (M = Molecular weight marker). (**b**) TEAD4 gene's exon/intron structure (exons are indicated by thick vertical lines of different colors; introns are indicated by thin horizontal lines), transcription, and splicing processes involved in the generation of the TEAD4-ΔN isoform. Pr-F and Pr-R indicate the position of the PCR primers used in the amplification of the novel transcript.

2.3. The Novel TEAD4 Isoform Encodes a DNA-Binding-Domainless Protein

In the next step, the coding capacity of the novel TEAD4 mRNA variant was investigated. We detected an open reading frame that encodes a truncated TEAD4 isoform lacking a DNA-binding domain (Figure 3). Consequently, the absence of the TEA/ATTS DNA-

binding domain results in compromised DNA-binding capacity of the encoded TEAD4 isoform. This therefore suggests that this isoform may have a unique cellular function.

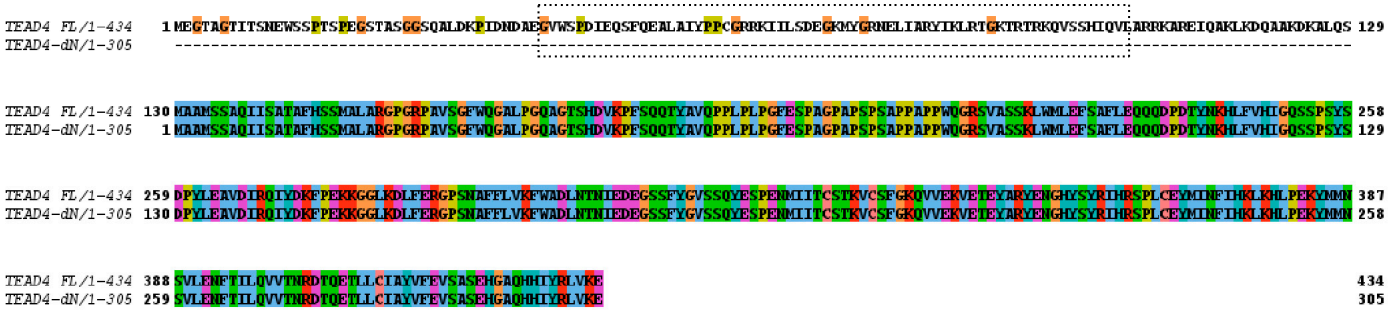

Figure 3. Pairwise protein-sequence alignment of TEAD4 isoforms. TEAD4-FL—amino acid sequence of the full-length TEAD4 protein. TEAD4-ΔN—amino acid sequence of the N-terminal DNA-binding-domain-deleted isoform. The DNA-binding domain is shown by the dotted frame, which is missing from the new TEAD4 isoform.

2.4. The TEAD-ΔN Isoform Is Excluded from the Nucleus

Several mechanisms have been described for regulating the subcellular localization of proteins. For instance, the nuclear localization signal (NLS) of NF-κB is covered by specific inhibitory proteins that prevent its constitutive entry into the nucleus [27]. However, proteins with no NLS can still fulfill their regulatory function through NLS-independent nuclear localization in cells [28]. The removal of the DNA-binding domain poses an interesting question about the cellular and molecular function of the TEAD-ΔN isoform. NLS prediction software (NLStradamus 1.0) could not detect this kind of sequence in the truncated TEAD4 isoform. Experimental evidence was sought to determine the location of the isoform. Our hypothesis was that identifying the subcellular localization of this isoform could provide insight into its potential function. Therefore, both isoforms were cloned into plasmids encoding fluorescent proteins. The full-length TEAD4 isoform (TEAD4-FL) was cloned into a plasmid expressing GFP (green fluorescent protein), while TEAD4-ΔN was cloned into a plasmid expressing RFP (red fluorescent protein). The recombinant plasmid constructs were transiently co-transfected into eukaryotic cells. The full-length isoform of TEAD4 (TEAD4-FL) was found exclusively in the nuclei, whereas the truncated form (TEAD4-ΔN) was mainly present in the cytoplasm (Figure 4). The cytoplasmic location of the isoform with a truncated DNA-binding domain may affect the regulation of the Hippo signaling pathway, as discussed later.

2.5. TEAD4-ΔN Expression Is Cell-Type-Specific

Cell-type-specific gene expression of the TEAD4 isoforms was investigated in total RNA samples isolated from various normal human tissues (Figure 5). Isoform-specific end-point PCR was used to investigate the gene expression of both isoforms in parallel. The results showed that the full-length isoform of TEAD4 was expressed in all the investigated tissue samples. In contrast, TEAD4-ΔN gene expression was found to be specific to certain cell types, suggesting a more intricate role for this novel isoform.

Western blotting was performed to determine the protein expression pattern of the TEAD4 isoforms in stable cell lines (Figure 6). Like the PCR studies, we detected the long isoform in all tested cell lines, whereas the short isoform was only present in specific cell lines, with significantly lower expression levels compared with the long isoform. The RNA- and protein-based data demonstrate isoform-specific differences in expression levels and distribution for the truncated TEAD4 isoform.

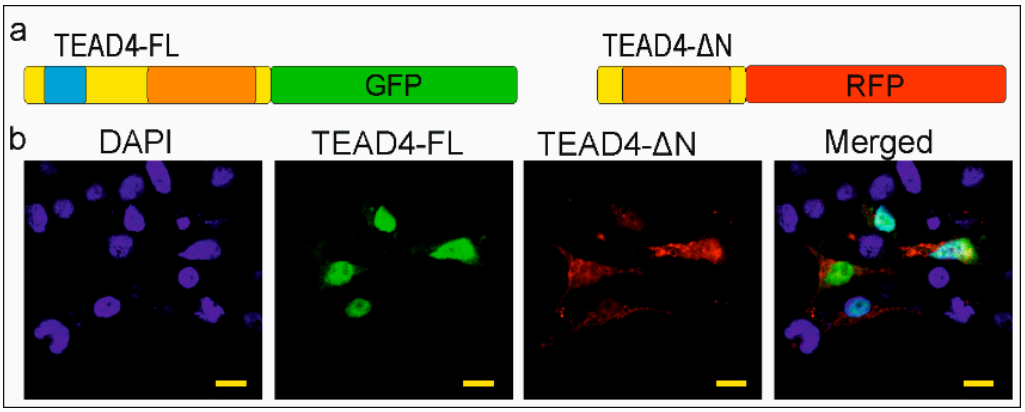

Figure 4. Subcellular localization of the TEAD4 isoforms. (**a**) Fusion constructs of the TEAD4 isoforms are depicted. The DNA-binding domains are highlighted as the light blue box, while the YAP-binding domains are represented by orange boxes. (**b**) The green fluorescent protein (GFP) and red fluorescent protein (RFP) are used as fusion tags. HEK293 cells were co-transfected with recombinant plasmids expressing either the full-length TEAD4 (TEAD4-FL) or truncated TEAD4 (TEAD4-ΔN) isoforms. Nuclear staining was performed using DAPI. The images were captured at a magnification of 800× with a scale bar of 10 μm.

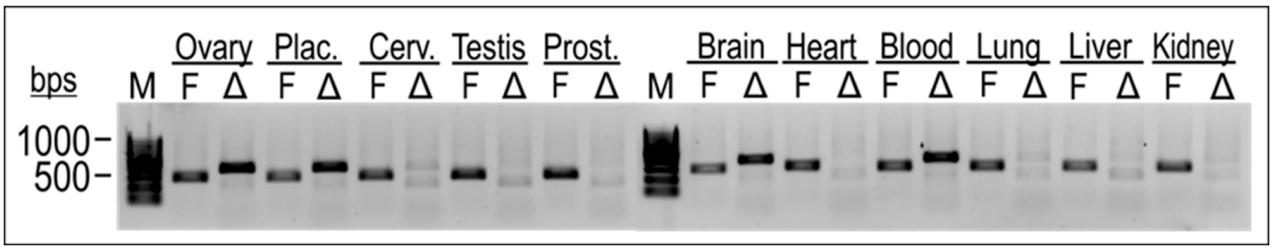

Figure 5. Isoform-specific end-point PCR was utilized to examine the expression of the TEAD4 isoforms in diverse tissue samples. RNA samples were reverse transcribed into cDNAs, and primer pairs specific for the full-length TEAD4 (TEAD4-FL) and truncated isoform (TEAD4-ΔN) were used in end-point PCR reactions. Amplicons were detected by agarose gel electrophoresis. F denotes TEAD4-FL, while Δ refers to TEAD4-ΔN. (M = Molecular weight marker).

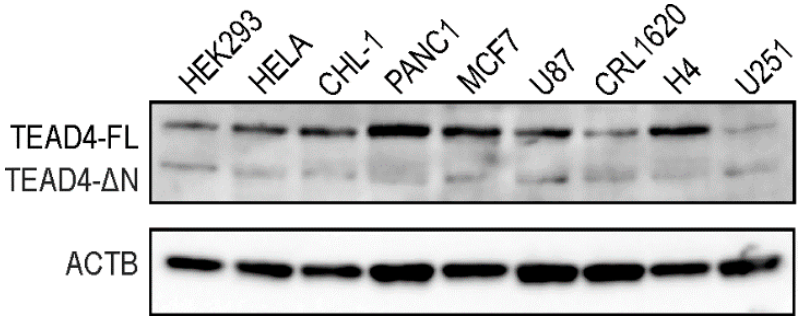

Figure 6. Expression levels of the TEAD4 proteins in cell lines. The expression pattern of full-length TEAD4 (TEAD4-FL) and truncated TEAD4 isoforms (TEAD4-ΔN) was detected by Western blotting. Human actin beta (ACTB) was used as a loading control.

2.6. In Vitro Analysis of the Alternative TEAD4 Promoter

Transcriptional regulation is based on the combinatorial binding of transcription factors (TFs) in the promoter and enhancer regions [29]. These bindings promote the formation of pre-initiation complexes and subsequent efficient transcription by the Pol2 enzyme. A search for TF binding sites in the TEAD4-ΔN promoter uncovered multiple potential binding sequences, including a consensus motif for TEAD4 itself, suggesting an interesting

self-regulatory mechanism. To confirm that the in silico predicted TEAD4 consensus (in the TEAD4-ΔN promoter) binds to this cis element, electrophoretic mobility-shift assays (EMSAs) were performed initially (Figure 7). As there were no commercially available high-quality antibodies for TEAD4 chips, we utilized a competitive EMSA approach to confirm TEAD4's binding to the novel promoter region.

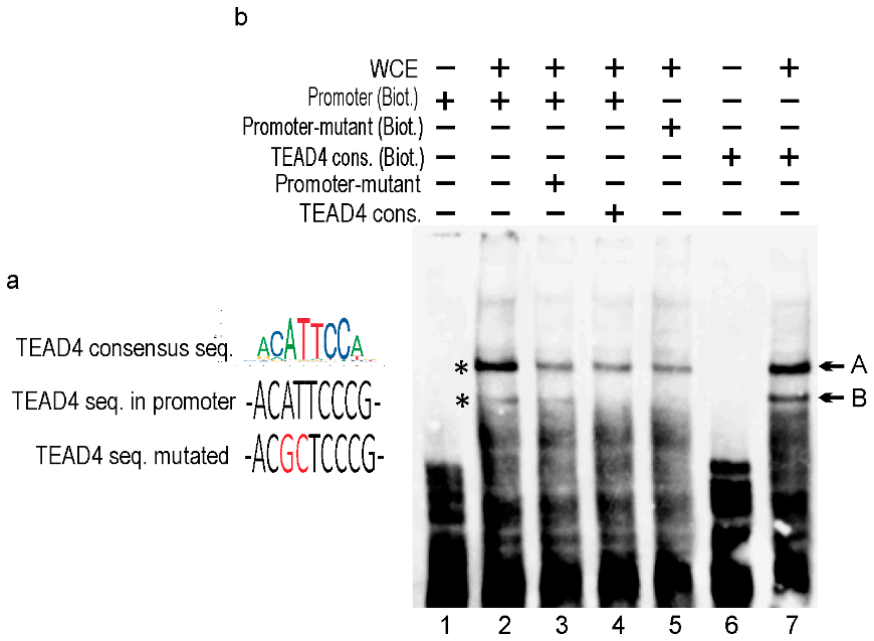

Figure 7. In vitro DNA–protein interaction study using EMSA. (**a**) The TEAD4 consensus motif is shown in color. The wild-type TEAD4 motif found in the TEAD4-ΔN promoter. The red label highlights the mutated core nucleotides in the TEAD4 binding site. (**b**) EMSA components included in each binding reaction are indicated using the +/− symbols. WCE—whole cell extract; Promoter (Biot.)—biotinylated promoter sequence containing the wild-type TEAD4 binding site; Promoter- mutant (Biot.)— biotinylated promoter sequence containing the mutant TEAD4 binding site; TEAD4 (Biot.)—biotinylated TEAD4 consensus sequence; Promoter-mutant and TEAD consensus—unbiotinylated double-stranded competitor oligonucleotides.

The nucleotide sequence of the predicted cis-element for TEAD4 binding is very similar to the consensus TEAD4 sequence [4] (Figure 7a). However, the core six nucleotides of the binding site are identical, differing in only one flanking nucleotide. A comparison of lanes 2 and 7 in Figure 7b demonstrates the binding efficency between the consensus and the actual TEAD4-ΔN promoter-related motifs. Accordingly, two TEAD4-specific bands were detected (labeled with asterisks) in EMSAs. The EMSA studies demonstrate that band B is exclusively formed by TEAD4 since the mutated promoter element lacks this band (Figure 7, lane 5), and competition with TEAD4 consensus oligonucleotide successfully eliminated band B (Figure 7, lane 4). TEAD4 might also be involved in the formation of band A; however, other interacting TFs are also shown to play a role in this DNA–protein complex formation.

2.7. Functional Characterization of TEAD4 Promoter(s) in Transient Transfection Studies

To gain more insight into the transcriptional regulation of TEAD4-ΔN expression and to evaluate the validity of the in vitro DNA–protein interaction studies, the promoter region upstream of the transcription start site (TSS) was amplified by PCR, and the obtained fragment was inserted into an expression vector upstream of the luciferase reporter gene. The 1.3-kilobase intron region was selected based on Ensembl Regulatory Build [30], a comprehensive database of epigenetic markers and transcription factors that provided a concise summary of potential TEAD4-ΔN regulatory regions (Supplementary Figure S2).

Additionally, the evolutionary conservation of this region suggests a complex role in transcriptional regulation (Supplementary Figure S3). Both the canonical TEAD4 and the TEAD4-ΔN promoters were cloned into recombinant luciferase-reporter-gene-containing plasmids. These plasmids were then transiently transfected into HEK293 cells to evaluate their activity in a eukaryotic milieu, as shown in Figure 8. In the assays conducted, the recently discovered promoter exhibited luciferase activity that was significant but less pronounced than the activity measured for the canonical promoter. This suggests that the isoform containing the DNA-binding domain may have the dominant cellular function, while the other isoform (TEAD4-ΔN) may only be necessary under specific conditions and in certain cellular environments.

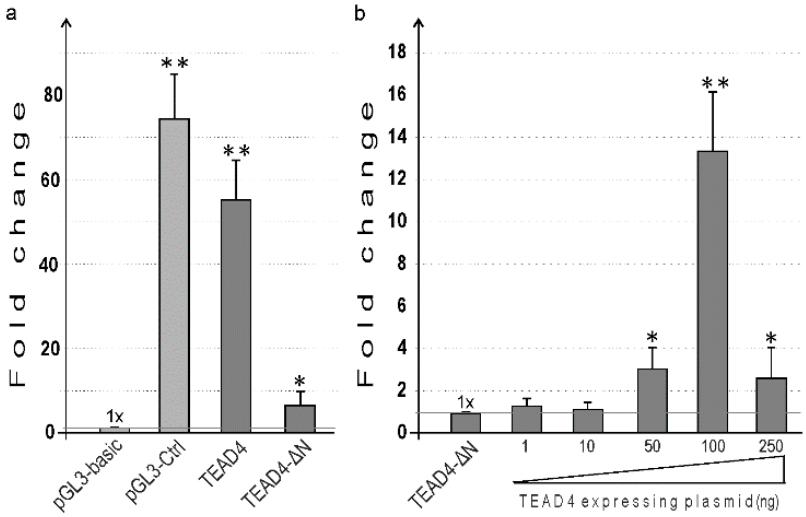

Figure 8. Functional characterization of the TEAD4 promoters in transient expression assays. Recombinant and control plasmids were transiently transfected, and luciferase activity was measured 48 h after transfection. (**a**) The TEAD4-ΔN promoter was functionally identified. The pGL3-basic plasmid was used as a promoterless control. The luciferase activity of the pGL3-basic plasmid-transfected samples was normalized to protein content and considered as 1-fold. pGL3-Ctrl: the plasmid used for transfection control is driven by the SV40 viral promoter and enhancer. There are two plasmids used for TEAD4: one carries a 1 kb long canonical promoter (TEAD4), while the other carries a 1.3 kb long intronic-region-inserted minigene construct (TEAD4-ΔN). (**b**) The effect of TEAD4 TF overexpression on the TEAD4-ΔN promoter-driven luciferase minigene. The luciferase activity of TEAD4-ΔN-plasmid-transfected samples was normalized to protein content (1-fold), and other luciferase activities were compared to that value. TEAD4-overexpressing plasmids were co-transfected at various concentrations with the TEAD4-ΔN reporter construct. Data sets show the mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$.

In vitro studies have shown that the TEAD4 transcription factor can engage the TEAD4 cis-element in the novel intronic promoter (Figure 7b). We assessed the impact of TEAD4 binding to this promoter region by co-transfecting a TEAD4-overexpressing plasmid with the reporter-gene construct carrying the 1.3 kb long TEAD4-ΔN promoter. By co-transfecting increasing amounts of a TEAD4-overexpressing plasmid, the promoter activity gradually increased, as demonstrated by increased luciferase activity. This implies that TEAD4 is a positive regulator of the expression of the truncated isoform of TEAD4 (Figure 8b). It is notable that high levels of TEAD4 can be inhibitory for promoter activity. Presented data sets in vitro and in vivo demonstrate that TEAD4 regulates the expression of its own isoform by interacting with its novel intronic promoter region.

2.8. TEAD4-ΔN Expression in Human Placenta Is Regulated by DNA Methylation

TEAD4 ensures the survival of human embryos after implantation by regulating the self-renewal and development of trophoblast progenitors in the placental primordium [13]. Research suggests that loss of TEAD4 in post-implantation TSPCs of mice diminishes their ability to self-renew, resulting in embryonic lethality before embryonic day 9.0, a developmental stage corresponding to the first trimester of pregnancy in humans [13]. Therefore, it is of great interest to determine how the expression of TEAD4-ΔN is involved in this developmental process. As an initial investigation, we analyzed the expression pattern of the TEAD4 isoforms in samples of human placenta and umbilical cord tissue (Figure 9a). We observed that the expression of the two TEAD4 isoforms was different in the analyzed human samples. Specifically, TEAD4-ΔN was not detected in the umbilical cord samples, while it was present in the placental lysates.

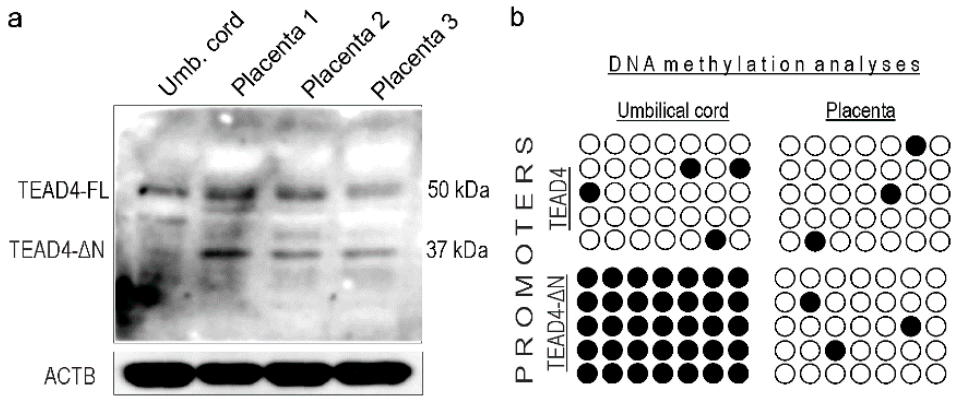

Figure 9. Protein expression and DNA methylation analyses were performed on human placental samples. (**a**) Western blot analysis of the expression of the TEAD4 isoforms in umbilical cord and three different placental samples. ACTB was used as a loading control. (**b**) Bisulfate sequencing analyses of TEAD4 promoters. Open circles represent unmethylated CpGs, while closed circles are methylated CpGs.

Epigenetic signals including DNA methylation and various histone modifications generate a chromatin milieu that sets the stage for efficient transcription. As a next step, we examined the probable epigenetic regulatory mechanisms underlying selective TEAD4 isoform expression. We conducted DNA methylation studies, including bisulfite sequencing, on genomic DNA samples isolated from umbilical cords and placentas. Our epigenetic studies were focused on revealing the DNA methylation status of the two TEAD4 promoters (i.e., the canonical and the alternative one), which might provide an explanation for the observed protein expression differences. The bisulfite sequencing data is consistent with the observed protein expression pattern. The canonical TEAD4 promoter is not methylated in either the umbilical or placental samples, allowing for uniform TEAD4 protein expression in these tissues. The promoter of the truncated isoform is heavily methylated in umbilical samples, resulting in impaired transcription, and, accordingly, there is no expression of the TEAD4-ΔN isoform in such samples, explaining the protein expression pattern detected by Western blotting.

3. Discussion

A new TEAD4-isoform-encoding transcript was identified, which is initiated from an alternative intronic promoter region. The TEAD4-ΔN transcript encodes an N-terminus truncated version of the well-characterized TEAD4 transcription factor. The novel transcript does not encode the DNA-binding domain (i.e., TEA/ATTS), suggesting that it is not directly involved in transcriptional regulation. This conclusion is supported by the observation that the TEAD4-ΔN:RFP chimera protein has no NLS and can be detected predominantly in the cytoplasm of transfected cells. A publication describes a short TEAD4

isoform, which is attributed to an alternative splicing event [31]. According to this report, the generation of the short TEAD4 isoform is associated with tumor-associated exon skipping, which is a rather rare event. We attempted to detect the corresponding mRNA produced by the alternative splicing event in various cell types but were unable to do so. However, we demonstrate that alternative promoter usage can also generate this variant with higher frequency, and it is not necessarily an erroneous splicing event associated with tumor formation. The TEAD4-ΔN transcript encodes the full-length YAP-binding domain, which may interact with the YAP1 protein in the cytoplasm and interfere with it. Phosphorylation of YAP-1 results in its translocation into the nucleus. Further investigation into TEAD4-ΔN-mediated regulation of the Hippo pathway may provide additional information on the finely-tuned mechanism involved in the differentiation of cell lines that affect embryo implantation in the uterus and placenta formation. Our study demonstrates that the expression of the TEAD4-ΔN-isoform-encoding mRNA is dependent on the DNA methylation status of the intronic promoter (Figure 10). The absence of DNA methylation may create a chromatin environment that promotes the decondensation of nucleosomes and attracts the binding of various transcription factors, including the DNA-binding-domain-harboring TEAD4. TEAD4 can form heterodimers with TEAD family proteins and SMAD TFs as well, increasing its significance in gene regulation [32]. During the pre- and post-implantation period of embryonic life, DNA-methylation-based epigenetic regulation is essential [33,34]. The DNMT3B enzyme is primarily responsible for de novo DNA methylation, which is necessary for regulating placental development and function [35]. The regulation of TEAD4-ΔN expression by DNA methylation raises intriguing questions about the molecular function of the truncated TEAD4 isoform. It is currently known that TEAD4 lacking a DNA-binding domain can disrupt the Hippo–YAP signaling pathway and interfere with cell proliferation, cell migration, and organ growth [31]. A short isoform of TEAD4 due to alternative splicing, known as TEAD4-S, has been reported [31]. It is highly possible that this isoform is identical to our TEAD4-ΔN at the protein level. In vitro studies have shown that TEAD4-S can inhibit the translocation of YAP to the nucleus and impair its interaction with transcription factors, including TEAD4. This can lead to remodeling of the whole transcriptome and disruption during tumorigenesis. We demonstrate here that occurrence of the short TEAD4 isoform is not necessarily associated with tumorigenesis; it can be expressed in a cell-type-specific manner from the newly discovered promoter as well. It is well known that DNA methylation can be heavily involved in the regulation of cell-type-specific gene expression [36], and in the preimplantation period of embryonic life, paternal and maternal DNA methylation patterns are erased and newly established [37]. Here, we have described a new TEAD4 isoform, the expression of which is tightly regulated by DNA methylation. According to a report, the short isoform of TEAD4 may impede TEAD4-mediated gene activation in cancer cell lines [31]. We observed that the promoter region of TEAD4 could be heavily methylated. Therefore, the use of DNA methyltransferase inhibitors (such as decitabine or azacitidine) may be a potential treatment option for certain tumor types [38]. In some tumors, TEAD4-ΔN may be overexpressed, making its silencing potentially beneficial. Treatment with DNA demethylase inhibitors, such as TET inhibitors, could be advisable [39]. Therefore, a systematic analysis of the DNA methylation status of the TEAD4-ΔN promoter in tumor samples can have diagnostic significance and provide guidance for treatment options.

Information on TEAD4-ΔN expression in preimplantation embryos (i.e., blastomeres) is currently unavailable. Therefore, it is unclear how TEAD4-ΔN expression can affect subsequent regulatory mechanisms. Although there are significant differences between human and mouse ontogenesis, employing an animal model can shed light on basic processes [40]. Accordingly, the evolutionary conservation of the TEAD4 promoter allows for the investigation of these processes in mice. TEAD4 and YAP are also involved in stem-cell renewal, which is essential during placenta development and for sustaining a functional placenta. Targeting TEAD4-ΔN in endometrial implantation and the subsequent placenta formation could have practical therapeutic significance, which might differ in

an oncology context. This is because current DNA methyltransferase inhibitors and TET inhibitors may cause mutations that lead to malformations during embryonic development. A new generation of DNA methylation inhibitors is on the horizon, which may increase their potential use for implantation-related issues [38] in the future.

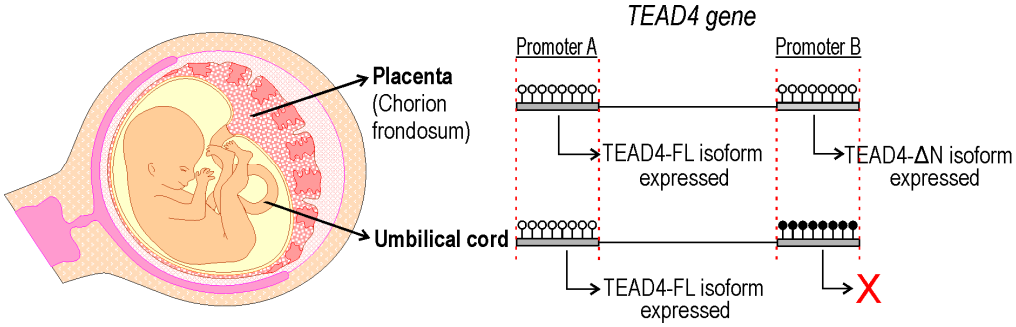

Figure 10. Epigenetic regulation of TEAD4 isoform expression in the placenta and umbilical cord. DNA methylation controls TEAD4 isoform expression. Promoter A, responsible for the expression of the full-length TEAD4 (TEAD4-FL), is unmethylated in all tissues, providing the constant presence of this isoform, while promoter B, responsible for the expression of the truncated isoform (TEAD4-ΔN), undergoes tissue-specific methylation, resulting in the tissue-specific presence of this isoform. Lollipops denote CpG dinucleotides: open ones are unmethylated; black-filled ones are methylated.

The molecular function of TEAD4-ΔN and its interacting partner proteins is not yet fully understood. Further research is needed to reveal the underlying mechanisms, which could have even more therapeutic and diagnostic implications.

4. Materials and Methods

4.1. Epigenetic Data Analysis

Histone-mark-related chromatin-immunoprecipitation data sets (i.e., ChIP-Seq data) were obtained from the ENCODE database (access date: 12.12.2023) [41]. The University of California Santa Clara, CA, USA maintains a publicly available source of ChIP-Seq research data.

4.2. Cell Culturing

The K562 (ATTC CCL-243), HEK293T (ATCC CTL-3216), and glioblastoma cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum. The cell cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 in air.

4.3. Total RNA Isolation

Total RNA was prepared from cell cultures using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA). RNA preparation from human samples was approved by the Ethics Committee of the University of Pécs (Code: 3648—PTE 2020, Epigenetic and Transcriptional Factors Involved in Placental Development). Additional total RNA samples used in this study were obtained from the FirstChoice® Human Total RNA Survey Panel (ThermoFisher Scientific, Waltham, MA, USA).

4.4. 5′RACE (5′ Rapid Amplification of cDNA Ends)

The 5′RACE method was utilized to determine the transcription start site of the uncharacterized RNA transcript [26]. 5′RACE was performed on 1 µg of total RNA isolated from K562 cells, resulting in a cDNA copy of the RNA sequence of interest. The 5′ end was amplified using anchor- and gene-specific primers, and the resulting fragment was visualized on an agarose gel. Subsequently, the amplified fragment was cloned into the pDrive plasmid (Qiagen, Hilden, Germany), and the nucleotide sequence was determined

by Sanger sequencing. The second-generation 5′/3′ RACE kit (Sigma-Aldrich, Saint Louis, MO, USA) was used to identify the start site of the TEAD4-ΔN-encoding mRNA transcript.

4.5. Nucleotide Sequence Analysis

Recombinant plasmids purified from bacteria were sequenced (Sanger sequencing) at the Department of Medical Genetics (University of Pécs), and "DNA Blat v23" software was used to identify the corresponding human genomic region.

4.6. Confocal Fluorescent Microscopy

To investigate the cellular localization of the two TEAD4 isoforms, we synthesized the corresponding ORFs in vitro (IDT, Coralville, IA, USA) and cloned them in-frame with green fluorescent protein (pEGFP-N1)- or red fluorescent protein (pDsRED-monomer-N1)-expressing mammalian expression vectors. We verified the correct fusions of the coding regions using Sanger sequencing. Recombinant plasmids carrying the full-length TEAD4-FL-GFP isoform and the N-terminal-truncated TEAD4-ΔN isoform were purified from bacteria and used in transient co-transfection studies. HEK293T cells were plated 18–24 h prior to transfection, and cell cultures were required to be at least 80% confluent at the time of transfection. The plasmids were combined in a 1:1 ratio, and the 293Tran transfection reagent (OriGene Technologies, Rockville, MD, USA) was used for co-transfection. After 48 h of transfection, HEK293T cells were fixed with paraformaldehyde and their nuclei were stained with 4,6-diamidino-2-phenylindole. The co-transfection efficiency was approximately 30%. Images were captured using a Zeiss LSM 700 confocal microscope and analyzed with Zen 2. software v2.2 (Zeiss). The slides were examined at a magnification of 800×.

4.7. Luciferase Reporter Assay

The promoter regions of TEAD4 and TEAD4-ΔN were amplified by PCR and cloned upstream of the luciferase reporter gene into the XhoI-HindIII sites of pGL3-basic plasmids. The recombinant plasmids were purified from bacteria using the ZymoPURE—Express Plasmid Midiprep kit (Zymo Research, Irvine, CA, USA), and 1 μg was transfected into HEK293 cells using the GenJet™ in vitro DNA transfection reagent. Cells that were transfected were harvested 48 h later, and luciferase activity was measured using the ONE-Glo™ Luciferase Assay System (Promega, Madison, WI, USA). The luciferase activity was normalized to the protein content, and the relative fold-change was calculated by considering the measured luciferase activity to be 1 in empty pGL3-basic-transfected samples. The untagged TEAD4-expressing plasmid was purchased from Origene (Rockville, MD, USA). Co-transfection was performed using 1, 10, 50, 100, and 250 ng of plasmid along with 1 μg of the TEAD4-ΔN-promoter–luciferase reporter plasmid. The amount of transfected DNA was kept constant at 2 μg by adding pUC18 plasmid. The relative fold-change was calculated based on the measurement taken from samples transfected with the TEAD4-ΔN-promoter–luciferase reporter plasmid only.

4.8. DNA Methylation Analysis—Bisulfite Sequencing (BS) [42]

The genomic DNA was prepared using the Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) from umbilical cord and placenta tissue samples obtained from the Department of Obstetrics and Gynaecology at the University of Pécs. Genomic DNA preparation from human samples was approved by the Ethics Committee of the University of Pécs (Code: 3648—PTE 2020, Epigenetic and Transcriptional Factors Involved in Placental Development). The isolated DNA samples were then treated with bisulfite using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA). MethPrimer software v.2.0 was used to design primers for the BS. PCR-amplified promoter regions were cloned into the pDrive vector. Plasmids were purified from 5 bacterial colonies, and the methylation status of CpGs was determined by Sanger sequencing.

4.9. Western Blotting

The cells on a confluent plate measuring 100 mm were lysed using M-Per mammalian protein-extraction buffer (Thermo Scientific, Waltham, MA, USA) supplemented with a protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Sigma) cocktail. The umbilical cord and placenta tissue samples were obtained from the Department of Obstetrics and Gynaecology at the University of Pécs. Protein-extract preparation from human samples was approved by the Ethics Committee of the University of Pécs (Code: 3648—PTE 2020, Epigenetic and Transcriptional Factors Involved in Placental Development). Tissue samples were homogenized in M-Per mammalian protein-extraction buffer (Thermo Scientific) supplemented with a protease inhibitor (Roche) and phosphatase inhibitor (Sigma) cocktail using a Dounce homogenizer. The resulting lysates were loaded onto 12% SDS–polyacrylamide gels and transferred onto PVDF membranes (Amersham). Membranes were blocked in 5% milk and incubated overnight at 4 °C with primary antibodies against TEAD4 (1:200 final dilution; Thermo Scientific) and β-Actin (1:1000 final dilution; Cell Signaling). Horseradish peroxidase-conjugated secondary antibodies specific to the species were used at a final dilution of 1:2000 (Cell Signaling). The immunocomplexes were visualized using Immobilon ECL Ultra Western HRP Substrate (Merck) and a Syngene G:BOX Chemiluminescence and Fluorescence imaging system (Syngene). Results were analyzed with GeneSys software v.2.1 (Syngene). Bio-Rad Precision Plus Protein TM Standards Kaleidoscope TM was used as molecular-weight markers.

4.10. Electrophoretic-Shift Essay (EMSA)

To identify and characterize protein–DNA-binding interactions associated with the TEAD4-ΔN promoter, we used the LightShift EMSA kit (Thermo Scientific) and followed the suggested protocol. The biotinylated oligonucleotides are listed in Table S1. The protein extract for the EMSA was prepared from 80% confluent HEK293 cell cultures as previously described [43].

4.11. Statistical Analysis

At least three independent experiments (triplicates) were conducted for all presented data. Data in the figures represent the mean ± SEM. Statistical differences were determined using the paired Student's *t*-test or One-Way ANOVA with Tukey HSD and Mann–Whitney tests. The specific differences were considered statistically significant if $p < 0.05$. Statistical significance is indicated by asterisks as follows: $p < 0.05$ *; $p < 0.01$ **.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

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