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Alternative Splicing of *TAF4*: A Dynamic Switch between Distinct Cell Functions

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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INTRODUCTION

Core transcription complex, consisting of RNA polymerase II and the cohort of general transcription factors (named GTFs or TFIIs), participates in all basal and cell-specific transcriptional processes. Its composition is not invariant and stable, but depends on the cellular context and executed molecular programmes. Various subunits and their paralogues engage in the transcription or are excluded from the process during the entire life cycle of the cell. Additional capability to diversify the already complex and poorly understood mechanism of cell-specific transcription is assigned to alternative splicing. Currently, there is not much data about the involvement of alternative splice variants encoding isoforms of GTFs into regulation of transcription due to their low availability, high diversification, complicated dynamics, and extreme complexity for investigation by modern methods. The biological role of alternative splice transcripts of GTFs is not completely understood. Only some examples of active alternative isoforms are described, such as apoptotic-specific TAF6δ (Bell et al., 2001). From the other side, effects of alternative splice variants of GTFs on transcription regulation could also be associated with their roles on the RNA level, acting as regulatory long non-coding RNA, or by other, as-yet undefined mechanisms.

TAF4, one of the basic and structural components of the TFIID complex, plays important roles in co-activator transcription, cell cycle regulation, and stability of the pre-initiation transcription complex (Wright et al., 2006; Layer & Weil, 2013). Its effects on cellular differentiation and cancer progression using gene silencing or overexpression approaches have been studied in some murine and human models (Metsis et al., 2001; Brunkhorst et al., 2005; Mengus et al., 2005; Fadloun et al., 2007; Ribeiro et al., 2014). Previously, it was shown that *TAF4* is subjected to intensive alternative splicing, with probable dominantly negative outcome in function of alternative isoforms in the mouse brain and neurons (Brunkhorst et al., 2004). To combine all previous achievements, we decided to investigate alternative splicing of *TAF4* in various human tissues and cells and assess its consequences on TAF4 function in normal cellular processes and in pathology. The results obtained provide more detailed insights into the understanding of the cell-specific functions of TAF4 depending on the cellular contexts.

ABBREVIATIONS

ASV - alternative splice variant
ATRA - all trans retinoic acid
BAF45/BAF53 - BRG1-Associated Factor 45/53
bHLH - basic helix-loop-helix
BMP - bone morphogenetic protein
CA150 - co-activator of 150 KDa
cAMP - cyclic adenosine monophosphate
CD - cluster of differentiation
CDK2 - cyclin-dependent kinase 2
ch-ERG - chicken *Ets*-related transcription factor
CREB - cAMP response element-binding protein
c-Jun - V-jun avian sarcoma virus 17 oncogene homolog
CTGF - connective tissue growth factor
DKK1 - Dickkopf–related protein 1
EGF - epidermal growth factor
EGFR - epidermal growth factor receptor
EMT - epithelial-mesenchymal progression
ESC - embryonic stem cell
ETO domain - 8;21 translocation domain
FGF - fibroblast growth factor
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
GTF - general transcription factor
HCF-1 - host cell factor 1
HFD - histone-fold domain
HP - heterochromatin protein
IGF - insulin-like growth factor
iPSC - induced pluripotent stem cell
KLF4 - Kruppel-like factor 4
KRAB - Krüppel associated box
KRT14 - keratin 14
lncRNA - long non-coding RNA
LZIP - leucine zipper protein
MMP3 - matrix metalloproteinase 3
MEFs - mouse embryonic fibroblasts
MITF - microphthalmia-associated transcription factor
MIA/CD-RAP - melanoma inhibiting activity/cartilage-derived retinoic acid-sensitive protein
MSC - mesenchymal stem cell
MYC - myelocytomatosis viral oncogene
NCoR - nuclear receptor co-repressor
NHNP - normal human neural progenitor
NMD - nonsense-mediated decay

NRSE - neuron-restrictive silencer element
OCT4 - octamer-binding transcription factor 4
OMA-1/2 - overlapping cctivity with M-AAA protease 1/2
OPN - osteopontin
ORO - Oil Red O
p300/CBP - p300/CREB-binding protein
PBAF - polybromo-associated BAF
PBX - pre-B cell leukemia transcription factor
PGC-1 - peroxisome proliferator-activated receptor-gamma coactivator
PIC - transcription preinitiation complex
PPARG - peroxisome proliferator-activated receptors
RARα - retinoic acid receptor-alpha
RNAi - RNA interference
RT-PCR - reverse transcription polymerase chain reaction
RT-qPCR - reverse transcription quantitative polymerase chain reaction
RXR - retinoid X receptor
SAGA - Spt-Ada-Gcn5 acetyltransferase, histone acetyltransferase complex
ShcA - Src homology 2 domain containing transforming protein A
snRNPs - small nuclear ribonucleoproteins
SWI/SNF - SWItch/Sucrose NonFermentable
siRNA - small interfering RNA
Sox9 SRY (sex determining region Y)-box 9
SKIP - Ski-interacting protein
Sp1 - specificity protein 1
SPT - suppressor of Ty protein
STAGA - SPT3-TAF9-GCN5 chromatin-acetyltransferase transcription coactivator complex
TAF - TATA-box protein associated factor
TAT-SF1 - cofactor required for Tat activation of HIV-1 transcription
TBP - TATA-box protein
TGFβ - transforming growth factor beta
TFIID - general transcription complex IID
TRF - TBP-related factor
TRRAP - TRAnsport Protein Particle complex
TYR - tyrosinase
TYRP1 - tyrosinase-related protein 1
VEGF - vascular endothelial growth factor
Wg/Wnt - family of wingless-related proteins
WNT5A - wingless-type MMTV integration site family, member 5A
WST – water-soluble tetrazolium salts
ZF - zinc finger

REVIEW OF THE LITERATURE

1.1 General principles of the transcription initiation

Transcription is main paradigm of molecular biology while double-stranded DNA gives raise a single-stranded RNA molecule, launching complicated biological processes. In one case, RNA molecule is "self-sufficient" and functions by itself; but in most cases, it followed by translation and results a protein molecule, the main player in the biological life marathon.

The two key stages in the transcription process are transcription initiation and RNA processing and synthesis (Nikolov & Burley, 1997; Hampsey, 1998). The transcription begins from attaching RNA polymerase II (Pol II) to the specific place on the template DNA strand, promoter, starting synthesis of complementary RNA (Thomas & Chiang, 2006; Kornberg, 2007; Baumann et al., 2010; Shandilya & Roberts, 2012). This is a crucial event that determines whether or not the gene is expressed. In addition to promoter, many eukaryotic genes possess enhancer sequences that are located at considerable distances from the target gene, controlling it by interaction with corresponding proteins and changing 3D structure and availability of DNA. The proteins that facilitate transcription in a positive way are called activators while those that inhibit it - repressors. Because eukaryotic DNA is tightly packed into condensed chromatin structures, transcription also requires a number of specific proteins to access DNA molecule and for full realization of Pol II activity (Ptashne & Gann, 1997). Although RNA Pol II is itself a large enzyme composed of multiple subunits, when active on DNA, it typically combines with other factors (Liu et al., 2011). Initiation of transcription is the first critical step in the regulation of gene expression. It requires simultaneous operation of a large cohort of transcriptional players – co-activators, trans-activators and components of the preinitiation complex (PIC), participating in the recognition of the core promoter (Tang et al., 1996; Kuras & Struhl, 1999). However, the general transcription machinery that consists of RNA Pol II and the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH is necessary and sufficient for basal (core-promoter mediated) transcription (Fig. 1). After Pol II anchors to the double-stranded promoter DNA through GTF interactions, TFIIH promotes ATP-dependent promoter unwinding, and stabilization of the open complex state; Pol II moves to the transcription start site (TSS) and transitions into elongation mode.

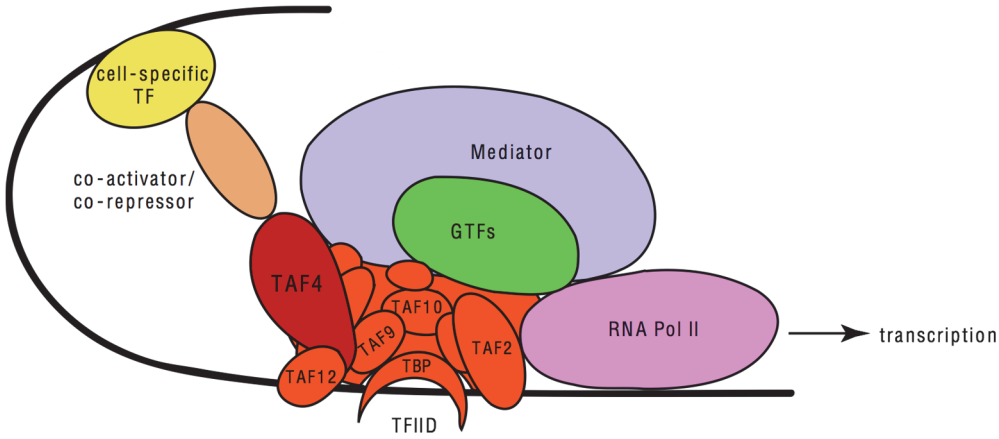

Figure 1. Multi-subunit tissue-specific RNA Pol II preinitiation complex at the site of transcription. The main components of basal transcription preinitiation machinery (RNA Pol II (lilac), GTFs (green), and Mediator (blue)) are presented. The tentative placement of some TBP-associated factors (TAF subunits) in the canonical TFIID complex (orange) is shown. TAF4 is specifically highlighted in dark red. The co-activators/co-repressors (light brown) bridge upstream DNA-bound cell-specific transcription factors (yellow) with transcription machinery. It should be noted that various PIC complexes with different subunit compositions and sequence variation (isoforms) do exist, thus further diversifying PIC architecture beyond the example shown in this figure.

In robust activator-dependent transcription, sequence-specific trans-activators bound to *cis*-regulatory elements (TATA, INR, DPE, MTE, etc.) of the DNA-template and impact PIC formation through TFIID and/or the Mediator complex by modification of chromatin structure and direct interaction with the core transcription machinery (Taatjes et al., 2004). As these core promoter factors are multi-subunit complexes, the size of the resulting PIC is greater than 2MDa. The operation of transcriptional regulatory machinery uses cooperativity of "basal" or "core" transcription subunits and upstream sequence-specific transacting factors (activators and repressors), using Mediator as a bridge for signalling transmission (D'Alessio et al., 2009). These master regulators control transcription of specific genes in a cell- and developmental stage-specific manner, affecting cell identity, growth and differentiation. The components of the "core" machinery are a conserved from yeast to human, while sequence-specific transcriptional factors vary significantly from organism to organism (Hager et al., 2009).

1.2 Diversity of TFIID and other TAF-containing complexes

The first clue that transcription apparatus is more complex than thought before, came in 1990s (Nikolov et al., 1992; Hernandez, 1993; Verrijzer et al., 1995; Arnone & Davidson, 1997). Namely, other components of large multi-subunit TFIID complex were discovered along with TATA-binding protein (TBP), participating in the core promoter recognition (Burley & Roeder, 1996). TBP-associated factors (TAFs) were biochemically identified as stably associated with TBP and originally named according to their electrophoretic mobility values (Dynlacht et al., 1991; Timmers & Sharp, 1991). Besides TBP, TFIID contains up to 14 different TAF subunits. The core composition of TFIID is relatively conserved from yeast to mammals. Interestingly, in addition to TBP, at least two TAF subunits (TAF1, TAF2) bind directly to the core promoter region in a sequence-dependent manner (Cianfrocco et al., 2013). Other components of the TFIID complex recognize multiple regulatory *cis*-elements in different combinations, as well as interact with modified nucleosomes and carry themselves different enzymatic activities (Hoffmann et al., 1997).

A modern vision of the transcription initiation includes one of its "general" factors, TFIID, that is structurally diverse and multi-component (Bieniossek et al., 2013; Demény et al., 2007). A functional core-TFIID sub-complex consists of a subset of TAFs such as TAF4, TAF5, TAF6 TAF9 and TAF12, which then become associated with peripheral subunits TBP, TAF1, TAF2, and TAF11 to assemble holo-TFIID (Wright et al., 2006). Additionally to canonical TFIID, tissue and developmental stage-specific forms of TFIID have been described. For instance, the presence of TAF10 in the TFIID complex is required for early development of the embryo, but not for later stages of development (Mohan et al., 2003). Besides canonical TBP, three additional TBP-related genes have been identified (Crowley et al., 1993; Rabenstein et al., 1999; Hansen et al., 1997). These are highly homologous proteins, albeit with distinct functional properties. Similar to the TBP-type factors, also some TAFs have paralogues that are expressed in the cell type- and tissue-specific manner. Thus, TAF1L and TAF7L are expressed during male germ-cell differentiation (Cheng et al., 2007), while expression of TAF4b is lymphocyte-, granulose-, ovary- and testis-specific (Freiman et al., 2002; Falender et al., 2005). Although the "peripheral" TAFs show restricted patterns of expression in different organisms, even the core TAFs appear at very different levels in certain cell types and tissues. Multiplicity of TFIID complexes allows a cell to exhibit functionally distinct properties and execute dynamic responses to cellular signals. Such as TAF4b-containing TFIID complex mediates transcription of genes required for folliculogenesis in ovary (Voronina et al., 2007), TFIID with alternative TAF6δ isoform and lacking TAF9 is necessary in responding to apoptotic stimuli (Wilhelm et al., 2010). The consequence of these observations is that structurally and functionally distinct forms of TFIID complexes participate in accession and recognition of various promoters, direct or indirect interaction

with different transcription factors to mediate diverse responses from activators/repressors to the PIC, for regulation of specific gene activation.

Dependent on the cellular contexts, TFIID serves as a target for various regulatory factors. In yeast, TFIID typically occupies TATA-less promoters (Hoffmann et al., 1997). Furthermore, in addition to TATA and TATA-less promoters, different core-promoter architectures have been identified, thus challenging the view of the PIC assembly been exclusively based on TBP sequence-specific binding complexes. Moreover, recent studies have shown that initiation of Pol II transcription can occur from TBP-free TAF complexes (TFTCs) (Hardy et al., 2002; Wieczorek et al., 1998). These are structurally similar to TFIID and contain in addition to TAF2, TAF4, TAF5, TAF6, TAF9, TAF12 and TAF6L some transcriptional co-activators, including the GCN5 histone acetyltransferase (HAT), TRRAP (a cofactor for Myc, E1A and E2F activation), and members of SPT and ADA protein families. The cooperation of TFTC with p300 has been described (Hardy et al., 2002).

Another large co-activator complex that binds TBP and has histone acetyl-transferase, ubiquitin-protease and transcription-activator activities is the yeast SPT-ADA-GCN5 acetylase co-activator complex SAGA (Grant et al., 1998). SAGA shares a subset of TAFs with TFIID, although structure and function of these complexes are different. SAGA is responsible for regulation of 10% of yeast genes, playing the role in TBP recruitment, although SAGA-TBP interaction is very weak (Yu et al., 2003). Up today, little is known of how SAGA fits into the PIC and associates with TFIID and Mediator.

Another class of multi-protein transcription regulatory complexes containing TAFs and having histone acetyltransferase activity, includes SPT3-TAF9-GCN5- containing STAGA complex (Wang et al., 2008), the p300/CBP associated factor PCAF complex (Schiltz et al., 1999), and GCN5 complex (Nagy & Tora, 2007). All of these complexes contain homologues of the yeast histone acetyltransferase (HAT) GCN5, SPT and ADA proteins, TRRAP adapter proteins, and a number of TAFs also found in the TFIID.

All these findings point out to the complexity of the eukaryotic gene regulation, recruiting various molecular processes involved in different cell functions.

1.3 Cell-specific transcription complexes

In early days, the common understanding of general transcription machinery was that it consists of the invariable core promoter recognition complexes, chromatin modifiers and co-activator proteins that through interaction with DNA-binding transcription factors control tissue-specific transcription (Müller et al., 2007). This view is now seriously challenged by a series of observations. The current understanding of transcription initiation mechanisms proposes that

a nearly infinite number of complexes composed of GTFs, Mediator and SWI/SNF subunits control gene expression in a cell- and tissue-specific manner. The modern collapse of the old "general" dogma is a result of parallel developments in proteomics, bioinformatics analysis of promoter regions and the application of new technologies, such as large-scale sequencing and one-cell analysis to description of transcription.

It is known that different genes use different combinations of core promoter elements, underscoring the more complex picture as that of the simple TATA versus TATA-less world (Ohtsuki et al., 1998). The universality of TBP was already questioned when TBP-related factors, including TRF1, TRF2 and TRF3, were discovered (D'Alessio et al., 2009). TRF1, which is highly homologous to TBP, represents a subunit of an alternative core promoter complex that in *Drosophila* directs promoter-selective transcription (Crowley et al., 1993; Hansen et al., 1997). TRF2, although broadly distributed from *C. elegans* to human, does not bind to TATA box-containing DNA and is unable to form canonical TFIID complexes (Rabenstein et al., 1999; Maldonado, 1999). It was demonstrated that TRF2 is required for early embryonic development (Dantonel et al., 2000) and is highly expressed in mouse testis (Sugiura et al., 2003). These observations imply that only a limited set of subunits, such as TBP and TRF2, is needed to produce highly varying expression patterns. The most intriguing and recently identified member of TBP-related family is the vertebrate–specific TRF3 (TBP2). It is widely expressed in the most adult mammalian tissues, but its role is crucial for oogenesis and during early embryonic development (Gazdag et al., 2007). Developmental studies of *Xenopus* have demonstrated that TRF3 can partially rescue the loss of TBP, suggesting different activation mechanisms rather than recruitment of specific transcription complexes (Jallow et al., 2004). TRF3 is essential for the initiation of hematopoiesis (Hart et al., 2009) and together with TAF3 is required for differentiation of myotubes (Deato et al., 2008).

Additionally to core TAFs, TAF paralogues are expressed in different cells and tissues (Hochheimer & Tjian, 2003). The majority of non-prototypical TAFs is detected during germ cell differentiation. For example, germ cell-specific TAF4b (Falender et al., 2005) that was initially discovered as B-cell-specific, and TAF7L (Pointud et al., 2003) that is involved in oogenesis and spermatogenesis, share similar domain structures with respective core TAFs. Association of TAF7L and TBP governs adipogenesis through the binding with PPARG-RXR cofactors and directing adipocyte-specific differentiation (Zhou et al., 2013). Five homologues of canonical TFIID subunits are expressed during spermatogenesis in *Drosophila*. Namely, the Cannonball, homologue of TAF5; No hitter, homologue of TAF4; Meiosis I arrest, homologue of TAF6; Spermatocyte arrest, homologue of TAF8; and Ryan express, homologue of TAF12 (Freiman, 2009). As a rule, up-regulation of expression of a paralogue leads to the dynamic down-regulation of its core partner with accompanying changes in TAF sub-complex structure and composition.

The most widely characterized eukaryotic chromatin-modifying complexes studied to date are ATP-dependent complexes, containing the SWI2/SNF2 and the imitation SWI (ISWI) groups, HAT and histone deacetylase (HDAC) complexes. They also exhibit considerable component diversity. Expression of BAF45 and BAF53 subunits of SWI/SNF complex is switched during neuronal maturation by a micro-RNA-dependent mechanism (Ho & Crabtree, 2010; Krasteva et al., 2012). PBAF chromatin remodeler complexes are different in cardiac progenitors and mature heart tissue (Wang et al., 2004). The most dramatic changes in the subunit composition of chromatin modifiers occur during embryonic stem cells (ESCs) development. A unique esBAF complex is essential for maintenance of pluripotency and self-renewal of ESCs (Ho et al., 2009); whereas disruption of BAF-A and BAF-B functional activities is required for lineage-specific differentiation of ESCs (Yan et al., 2008).

Mediator complex complements tissue-specific transcription as an adapter element between RNA Pol II machinery and upstream transcription factors. Mediator is a 26-subunit complex that is specific to the eukaryotes only. Some individual subunits of Mediator are regarded as invariable complex components for the development of metazoans (Taatjes et al., 2004). Other subunits are considered as activator-specific. Consistent with this, MED1 interaction with MED24 serves as a cofactor for thyroid hormone signalling (Yuan et al., 1998). MED24 itself is necessary for the development and proliferative activity of neural progenitor cells (Pietsch et al., 2006). In turn, MED17 has a more general role in transcription initiation and in cell viability-related functions (Rachez & Freedman, 2001). Differential requirements for various subunits of Mediator complex have been described during zebrafish retinal development (Dürr et al., 2006). Consistent with this, MED12 is essential for SOX9 activity and its inactivation dramatically affects cartilage differentiation (Rau et al., 2006).

Despite emerging data on the diverse composition of general transcription complexes and their heterogeneity of components during development, very little is known of the orchestration of the basal transcriptional activity in tissue-specific differentiation.

1.4 The functional role of TFIID complex components in development

Given that the diversity of TFIID complexes is vast during development, their composition and functional dynamics at different stages of development are poorly understood.

The identity of ESCs is governed by a set of sequence-specific pluripotent transcription factors, including OCT4, MYC, KLF4 and NANOG (Chambers & Tomlinson, 2009). These factors control the activity of the basal transcription complexes via specific interactions with co-activators. Herein, TFIID activity

contributes the most to the induction and maintenance of the highly plastic pluripotent state of ESCs (Baumann, 2013). Recent studies in mouse ESCs revealed preferential binding of TFIID to nucleosomes with the active epigenetic H3K4me3 and H3K14ac marks (Nuland et al., 2013) and at genomic regions spanning transcription start sites characteristic to mouse ESCs (Ku et al., 2012).

At the earliest stage of vertebrate development, in the fertilized egg, two dynamic processes are highly crucial: zygotic gene activation and degradation of maternal mRNAs (Fig. 2). Both of these events are regulated by the TBP activity (Ferg et al., 2007). Up-regulation of nuclear TBP levels promotes zygotic gene activation and the ratio of nuclear to cytoplasmic TBP protein regulates gradual degradation of cytoplasmic maternal mRNAs. Discovery of TRF2 and TRF3, also detected in the early embryo, reinforced the importance of redundant functions of TBP for the initial stages of development. However, much less is known about the composition of TFIID complexes during zygotic development of vertebrates. One of the studied mechanisms observed in *C. elegans* is regulation of transcription initiation by sequestration of TAF4 to the cytoplasm (Guven-Ozkan et al., 2008). Dynamical changes in the basal transcription complexes have been characterized for TAF5, TAF10 and TAF11 subunits during zygotic gene activation (Walker et al., 2001). In two-cell stage, TAF5 was identified as vital for the open-complex formation, whereas TAF10 and TAF11 were detected in four-cell-stage nuclei with no impact on RNA Pol II activation (Walker et al., 2001). Similarly, in zebrafish embryos, both TBP and TAFs are highly expressed during early phases of gastrulation and their levels drop sharply at later stages of development (Tadros & Lipshitz, 2009). Strict requirements of TBP and several TAFs have been shown for the most of zygotic gene activation during mouse early development, highlighting the classic modes of transcription initiation by the canonical TFIID complexes.

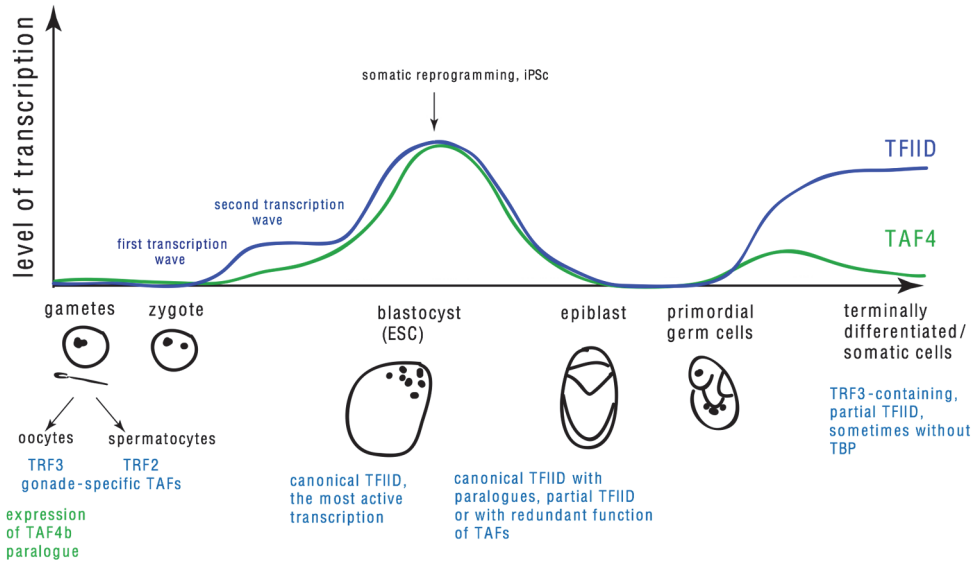

Figure 2. Schematic presentation of the activation dynamics of TFIID and TAF4 protein complexes during different stages of vertebrate development. Blue line shows change dynamics of TFIID complex activity from two-cells embryo to the terminally developed organism. Green line demonstrates the dynamics of TAF4 activation during development. Various stages of development and comments regarding the TFIID composition are shown under the scheme.

1.5 Opposing effects of TFIID subunits on cell differentiation

Previously it was suggested that the interaction of the basal transcription complex with a set of tissue-specific transcription factors is required for embryonic development and differentiation. However, more recent publications argue that lineage-specific differentiation involves the selective loss of some of the common RNA Pol II core complex subunits (D'Alessio et al., 2009). Besides, the expression of different TFIID subunits is comparatively lower in non-differentiated cells as compared to fast proliferating cells (Hochheimer & Tjian, 2003). Whether TFIID subunits are actively degraded and/or replaced by paralogues is currently not clear (Fig. 2).

Individual TAFs interact with upstream transcription factors and require specific core complex-subunits for conveying their functional activity. These core subunits are TAF4, TAF5, TAF6, TAF9 and TAF12; they present in two copies and form symmetric structure in functional TFIID core-subcomplex (Bieniossek et al., 2013). Thus, canonical TFIID complexes do not contribute to transcription of the majority of genes in terminally differentiated hepatocytes

(Tatarakis et al., 2008). Several TAFs interact with developmental transcription factors in the lineage-specific manner and require definite core partners for cooperation. Consistent with this, TRF3-TAF3 complexes drive hematopoiesis and myogenic differentiation (Hart et al., 2007; Hart et al., 2009), and are absolutely essential for endodermal lineage commitment (Liu et al., 2011); TAF4 controls ATRA-dependent differentiation (Fadloun et al., 2008), and TAF8 is involved in adipogenesis (Guermah et al., 2003). On the other hand, different transcription initiation mechanisms co-exist in cells during differentiation. Expression of some genes requires most of the canonical TFIID complex subunits, while other genes are transcribed by subsets of TFIID components. For example, expression of TAF8 is not detected in preadipocytes but is upregulated during adipogenic differentiation, when the expression of other TAFs is downregulated (Guermah et al., 2003). TAF10 is required for normal liver development (Goodrich & Tjian, 2010). Reduced expression of Taf1 and Taf4b affects proliferation of mouse embryonic maxillary mesenchymal cells and causes aberrant bone formation (Iwata et al., 2010).

Germ cells are the best-characterized model of the transcription machinery adaptation during differentiation (Hiller et al., 2001). So far, the most diverse set of TBP and TAF paralogues is found in germ cells, supporting the concept of specialized TFIID complexes that are distinct from the canonical forms. For example TAF4b, the first identified tissue-specific TAF, is important for ovarian follicle development and function (Dikstein et al., 1996). Male germ cell-specific TAF7L governs male fertility (Zheng et al., 2010). Expression of meiotic genes in gametes is controlled by TRF2 (Siderakis & Tarsounas, 2007). Oocyte-specific TRF3 replaces core TBP in these highly specialized cells, and its expression decreases during fertilization, being substituted by TBP (Müller & Tora, 2009).

On the whole, very different models of transcription initiation operate during embryonic development and in adult organism, supporting the concept of different transcription regulatory networks in proliferating and differentiating cells (Müller et al., 2010) (Fig. 2). Similar to early stages of development, in ESCs and pluripotent stem cells, active canonical TFIID complexes are required for supporting cell function. The majority of TAFs are expressed in ESCs at high levels. TAF3 and TAF4 are specifically required for maintaining their pluripotency (Liu et al., 2011; Pijnappel et al., 2013). In contrast, different TFIID sub-complexes are present in terminally differentiated cells, thereby reflecting the need of different cell types to respond to different external signals. High plasticity and adaptability of the core complex subunits allow both, highly specialized and broad initiation of transcription depending on the cellular context and developmental setup.

1.6 General principles of stem cell differentiation

The definition of stemness based on self-renewal of stem cells and their potential to differentiate into various cell lineages. According to the cell type origin, stem cells are classified into embryonic stem cells (ESCs), somatic stem cells (SSCs), and induced pluripotent stem cells (iPSCs). ESCs are derived from pre-implantation embryos before trophoectoderm and morula stage differentiation and capable of giving rise to all tissues (totipotent). However, ESCs derived from inner cell mass and having the ability to differentiate into all three germ layers but not placenta are pluripotent. iPSCs can be generated from somatic cells by variety of epigenetic and molecular biology methods and propose to have pluripotent nature, but not quite similar to the ESCs. The possibility of adult mammalian stem cells to differentiate into various tissues has been a widely studied area of research. Among others, mesenchymal stem cells (MSCs) are the example of plastic multipotent somatic stem cells that have the potential to differentiate to all mesenchymal cell lineages (Pittenger et al., 1999). Successful differentiation of MSCs into bone (osteoblasts), fat (adipocytes), cartilage (chondrocytes) and muscle (myocytes) has been described. Under certain culture conditions, MSCs are able to trans-differentiate to other than mesenchymal origin of cells like neurons, cardiomyocytes, etc (Wagers & Weissman, 2004).

Mainly, MSCs are derived from the stroma of bone marrow, but they may also be isolated from peripheral blood, umbilical cord, and adipose tissue (Lee et al., 2004; Zuk et al., 2002). Originally, MSCs were isolated from the bone marrow aspirate based on their feature to adhere to the plastic of the cell culture plate. MSCs are present in relatively low numbers and need to be enriched and expanded prior to use.

To distinguish mesenchymal stem cells from hematopoietic cells, a set of hematopoietic CD34, CD45 and CD14 markers is defined as not expressed in MSCs. For positive selection, in order to gain MSC enriched cell populations, the cell surface markers CD105 (endoglin), CD73 and CD90 are used (Dominici et al., 2006). However, there are no any MSC-specific cell surface markers that exclusively identify MSCs, and thereby all isolated MSC populations are still heterogeneous (Alhadlaq & Mao, 2004).

Due to their proliferative and multipotent differentiation potential, MSCs are a valuable tool for modern regenerative medicine and stem cell therapy. Thus, MSCs may be used as therapeutic strategies for *in vivo* transplantation after injury (Kang et al., 2012). Being extremely potential in damaged tissue repair, MSCs are used for cardiac recovery after myocardial infarction and for treating of liver diseases (Shake et al., 2002; Zhang et al., 2012). However, the exact mechanisms of repair have not been yet elucidated.

Regulation of transcription helps to establish differential gene expression profiles that confer cellular identity. Together with core transcription subunits, carefully controlled activity of transcription factors is responsible for realization of certain differentiation programs. Core transcription factors support basal levels of expression, whereas lineage- or developmental-specific transcription factors orchestrate spatio-temporal patterns of gene expression. 5-10% of total coding capacity of eukaryotic genomes belongs to transcription factors (Levine et al., 2014). Tight control of transcription regulatory activity is particularly important for stem cells because of the possibility of irreversible premature differentiation (Boyer et al., 2005).

1.7 Cancer stem cells as a cell source for multi-lineage differentiation

Due to the rapid development of regenerative medicine, research efforts have mostly focused on stem cells for replacing damaged tissues through new organ reconstruction.

The first direct evidence of the existence of cancer stem cells came from the discovery of leukemic hematopoietic stem cells in 1997 (Bonnet & Dick, 1997). Although cancer stem cells have not been identified in all tumours, they are present in brain, breast, colon, ovary, melanoma and other malignancies (Singh et al., 2003). Along these lines, multi-lineage differentiation of colon cancer stem cells has been demonstrated both, by *in vitro* and *in vivo* studies (Vermeulen et al., 2008). Small cell lung cancer cells with expression of neuroectodermal and mesodermal marker genes could be induced to differentiate into neurons, adipocytes, and osteocytes (Zhang et al., 2013). Among investigated tumours, glioblastomas have been shown to differentiate *in vitro* towards neural and mesenchymal lineages, and generate chondrocytes (Ricci-Vitiani et al., 2008).

The ability of cancer stem cells to self-renew and differentiate into various cell lineages is similar to the physiological properties of stem cells. On the other hand, formation of functional iPSCs from primary tumours has been successfully demonstrated by expression a set of upstream transcription factors (Carette et al., 2010), or reprogramming cellular epigenetic states by ectopic miRNAs (Lin et al., 2008). Generating of stem cells from neoplastic cells expands certainly the overall understanding of general principles of cellular pluripotency and differentiation. However, for cell therapy purposes, transformation of cancer stem cells into non-tumorigenic progeny is imperative due to their carcinogenic potential, unlimited growth and apoptosis resistance.

1.8 Regulation of protein function by alternative splicing and its functional consequences on cellular homeostasis

For the most eukaryotic genes, the initial RNA must be processed before it turns into a mature mRNA. One of the steps, involved in this transformation, is RNA splicing. Alternative splicing plays a key role in generating of complex proteomes, breaking "one gene, one protein" rule. Existence of multiple mRNA variants for a single gene explains, at least in part, the complexity of some organisms like humans, having about 20,000 protein-coding genes in their genome (International Human Genome Sequencing Consortium, 2004). Moreover, alternative splicing enables quantitative control of gene activity levels through regulation of expression of regulatory RNAs and by targeting RNAs to nonsense-mediated decay. Genome-wide analysis has revealed differential expression of alternative spliced mRNAs in various tissues and cell types (Castle et al., 2008).

According to the analysis of expressed sequence tag (EST) and cDNA data, it was estimated that alternative splicing affects the expression of nearly 90% of human genes (Modrek & Lee, 2002; Wang et al., 2008). Extremely complex alternative splicing patterns of *Drosophila Dscam* gene could give rise to 38,016 DSCAM proteins (Schmucker et al., 2000). High accuracy of alternative splicing is extremely crucial since about 20-50% of human diseases originate from mistakes in the splicing process (Cáceres & Kornblihtt, 2002).

The main principle of alternative splicing stems from the structure of eukaryotic gene, where exons that construct the mRNA are interrupted by non-coding introns dispersed throughout genomic DNA. In the canonical process, introns are removed from the primary transcripts by the cleavage, and the remaining exons are joined together by a macromolecular ribonucleoprotein complex - spliceosome (Jurica & Moore, 2003).

Alternative splicing could yield in different combinations of exon-joining events. Most alternative splicing events involve the coding exons, in some cases partially changing the open reading frame (Clark & Thanaraj, 2002), which in some cases may lead to the degradation of target mRNA through NMD due to premature termination codon generation (Lewis et al., 2003).

Consistent with the notion that alternative splicing avoids destruction of the functional protein domains, it targets mostly the areas of structure with minimally exposed hydrophobic surface and/or with high intrinsic disorder (Romero et al., 2006). Removal of protein-protein interaction domains by alternative splicing affects protein function and stability including misfolding and aggregation, capacity for protein interactions, appearance of isoforms with dominant-negative function. Of the total number of alternative splicing events, it is estimate that the functional domains are deleted at most in 10% of cases (Resch et al., 2004).

One of the interesting features of alternative splicing is the generation of alternative isoforms with opposite roles. For example, alternative splicing generates isoforms of nuclear receptor co-repressor NCoR that perform completely different functions during adipogenic differentiation (Goodson et al., 2011): NCoRδ promotes, while NCoRω isoform suppresses differentiation of adipocytes. Similarly, transcription factor OCT2 has two isoforms with dual, blocking and inducing, roles in neurogenesis (Theodorou et al., 2009). The opposite effects on chondrogenesis of different isoforms of transcription factor PEBP2 generated by alternative splicing are well described (Akiyama et al., 1999). Alternative splicing of *p63*, the member of the p53 transcription factor family, is highly complex yielding in different alternative p63 isoforms coordinating various steps of skeletal development (Gu et al., 2013).

Diversity and multiplicity of formed alternative RNAs raises questions about possible functional roles for the other 98% of the human genome that does not encode proteins. In fact, >90% of the human genome is likely to be transcribed (ENCODE Project Consortium, 2007). Resulting complex network of overlapping transcripts includes thousands of long RNA transcripts with little or no protein-coding capacity (Kapranov et al., 2007). To date, a lot of non-coding RNAs (ribosomal RNAs, transfer RNAs, and spliceosomal RNAs) are critical components of important cellular processes. It seems highly possible that non-coding transcripts have key regulatory and functional roles (Wilusz et al., 2009). Their possible function on molecular level may include for example modulation of protein-protein interaction.

One of the major challenges today is the understanding of the role of specific splice variants in a cell context. For that, various genetic and epigenetic techniques are applied. One of them is baced on siRNA silencing approach. It has been shown that targeting of intron or exon sequences near the alternative-splicing sites by respective siRNAs affects the splicing process of these sequences (Alló et al., 2009). Supposed mechanism involves transcription-coupled alternative splicing. Among protein isoforms whose function is studied by using this approach are adapter protein ShcA (Kisielow et al., 2002), spleen tyrosine kinase (Prinos et al., 2011), and pyruvate kinase M1 and M2 (Goldberg & Sharp, 2012).

As a rule, complicated regulation of gene expression in higher eukaryotes, in particular at the level of alternative splicing of transcription regulatory factors, provides significant advances for basic research and clinical applications.

1.9 Coupling of alternative splicing and transcription and functions of alternative isoforms of TFIID subunits in cell

To date, a close link between transcription and splicing has been found (Kornblihtt et al., 2004). Some transcriptions factors like TAT-SF1, CA150, SKIP and co-activator PGC-1 are present in the spliceosome and perform dual functions in transcription and splicing. Thus, it is highly conceivable that both processes are simultaneously coupled together in space and time (Proudfoot et al., 2002). Consistent with this, C-terminal domain of RNA Pol II directly participates in exon recognition (Zeng & Berget, 2000); identification of promoter structure is often associated with alternative splicing of pre-mRNAs (Cramer et al., 1997); some transcriptional co-activators and co-regulators modulate alternative splicing, sometimes in a synergetic manner (Nogues et al., 2002; Auboeuf et al., 2002).

Interestingly, changed by alternative splicing protein-protein interaction domains are very common to regulators of transcription (Taneri et al., 2004; Talavera et al., 2009). Ankyrin repeat, DNA-binding zinc finger, homeobox, and KRAB (Resch et al., 2004) domains are frequent targets of alternative splicing modification. Hormone-binding domains, presented in nuclear hormone receptors and PHD and involved also in interactions with chromatin, are frequent targets of alternative splicing (Talavera et al., 2009). In general, percentage of genes in human and mouse genomes affected by alternative splicing is higher for transcriptional factors than for any other protein group (Taneri et al., 2004; Talavera et al., 2009).

Interaction of splicing factors with regulatory elements in the pre-mRNA together with pace and pausing of elongation of transcription are important factors of the alternative splicing regulation. Chromatin context and intragenic DNA methylation affect both Pol II processivity and alternative splicing (Kornblihtt, 2006; Lorincz et al., 2004). Rapid, highly processive transcription supports exon skipping, while low-pace transcription leads to the exon inclusion.

Function of isoforms of different TFIID components generated by alternative splicing is not well understood. A pro-apoptotic TAF6δ isoform is one of the most investigated subunits of TFIID complex. It differs from the major ubiquitously expressed TAF6α isoform in 10 amino acids that are lost from its histone fold domain (Wang et al., 1997; Bell et al., 2001). As a consequence, modified TAF6δ protein cannot interact with its direct partner TAF9 or other TFIID subunits like TAF1, TAF5, TAF12 and TBP, and recruits a different TFIID-like complex, thereby altering global gene expression. TBP isoform with the polyglutamine-containing N-terminal domain only is detected in the human brain tissues and implicated in Alzheimer's disease (Reid et al., 2009). Inducible alternative splicing of *Taf1* in *Drosophila* is a mechanism that regulates transcription in response to developmental or DNA damage signals

(Katzenberger et al., 2006). It operates through ATR-signalled degradation of a subset of splicing-regulatory proteins (Katzenberger et al., 2009). In mouse, cell type-specific isoforms of TAF4 have been discovered (Brunkhorst et al., 2004). Some of them have dominant negative effects on nuclear receptor-mediated transcriptional activation. In addition, TAF9β, but not TAF9α isoform participates in megakaryocyte development through Mpl-ligand dependent mechanisms (Thompson & Ravid, 1999).

1.10 TAFs in cancer

For many years, the role of TAFs was thought as that of a signal transmitter from upstream activators to the basic transcription machinery. Their function was connected exclusively to the function of core transcription apparatus during normal cell homeostasis. However, recent studies suggest that TAFs are implicated in cell cycle regulation, apoptosis, and epithelial mesenchymal transition (EMT) (Davidson et al., 2005; Pijnappel et al., 2009). Deregulation of TAF activities leads to neoplastic transformation of cells, highlighting the importance of their balanced expression in cellular processes (Ribeiro et al., 2014). A bioinformatics study of 316 high-grade ovarian carcinomas revealed that TFIID is the most significantly changed complex in its subunit composition (Cancer Genome Atlas Research Network, 2011).

In addition, TAF4b has been identified to regulate a set of genes involved in EMT progression such as activating protein-1 (AP-1), c-Jun and integrin α6, thereby contributing to the migration potential of cancer cells (Kalogeropoulou et al., 2010). TAF4 affects TGF-β signalling, driving cells through EMT towards carcinogenic transformation (Mengus et al., 2005). TAF4 deficit reorganizes cells to produce pro-oncogenic Col6A3, preventing contact inhibition and promoting three-dimensional growth of MEFs (Martianov et al., 2014). TAF12 forms important for TFIID assembly heterodimer with TAF4 and participates in RAS-induced transformation of colorectal cancer cells (Voulgari et al., 2008). Interaction of TAF1 with retinoblastoma tumour suppressor protein Rb inhibits the kinase activity of TAF1 and represses transcription from specific promoters, possibly implicated in cancer progression (Siegert & Robbins, 1999). A genome-wide RNAi screen has established that TAF1 acts as an apoptosis regulator that is induced by genotoxic and oxidative stress (Kimura et al., 2008). Overexpression of TAF1 and its interactions with E2 of HPV affect E2-dependent transcription in cervical cancer (Centeno et al., 2008). Reduction of TAF7 levels suppress the transport of polyamines and cause resistance to apoptosis in androgen-independent prostate cancer (Fukuchi et al., 2004), while decreased expression of TAF7L is observed in 59% males with acute myeloid leukemia (Yazarloo et al., 2013). Overexpression of TBP alone could be transformative for cell, and its upregulation is detected in some colon cancers (Johnson et al., 2003).

In general, TAF-containing complexes besides their gene-specific regulatory functions and roles in phosphorylation and methylation processes have global chromatin modifying properties, participating in histone and transcription factor acetylation. Smallest changes in the balance between acetylation and deacetylation can lead to serious consequences of cell fate. The E2F family of transcription factors is a key cell-cycle regulator and misbalances in E2F activity can result in tumorigenesis. Notably, TAF4 (Chen et al., 2013) and TBP (Lang et al., 2001) have been found to be the direct targets of E2F activity, suggesting their involvement in carcinogenic outcome. It is well established that p53 is one of the most extensively studied tumour-suppressor proteins in mammals. Its activation leads to the cell-cycle arrest and/or apoptosis depending on the cell context. Functional association of p53 with TAFs has repeatedly been established (Farmer et al., 1996). Pro-apoptotic TAF6δ interacts with p53 and coordinates the induction of genes involved in apoptosis (Wilhelm et al., 2010), at the same time it also elicits p53-independent regulation (Wilhelm et al., 2008). TAF9 has been found to act as a crucial co-activator of p53 signalling (Lu & Levine, 1995; Jabbur et al., 2002; Buschmann et al., 2001).

Some of the TFIID components, TAF2, TAF3, TAF4 and TAF9 in particular, are important for maintenance of cellular pluripotency and regulation of differentiation. Their deregulated expression could contribute to cancer initiation and progression (Ribeiro et al., 2014).

1.11 Melanoma progression

Human malignant melanoma is probably the most metastatic cancer that is markedly resistant to conventional therapies. Melanomas arise from epidermal melanocytes, the pigment-producing cells of the skin. However, they can also derive from noncutaneous melanocytes (Chin et al., 2006). Among basic risk factors are the genetic predisposition and harmful UV radiation (Hussein, 2005).

During melanoma progression, fully differentiated melanoma cells leave the neighbourhood of keratinocytes by similar to EMT mechanisms, including downregulation of E-cadherin, upregulation of molecules important for cell-cell interactions such as N-cadherin, and loss of anchoring to the basement membrane in response to the altered adhesion profile (Haass et al., 2005). Distinct changes in malignant microenvironment cause activation of secretion of proliferative growth factors such as IGF1, FGF2, TGFβ or VEGF and modulate proliferation, invasion and migration of melanoma cells.

The most important step in melanoma progression is driven by the microphthalmia associated transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor and melanoma oncogene (Price et al., 1998; Steingrímsson et al., 2004). MITF plays an important role in

survival, differentiation and pigmentation of melanocytes. It has been shown that expression of MITF alone is sufficient to convert ES-like cells and fibroblasts into cells with characteristics of melanocytes (Béjar et al., 2003; Tachibana et al., 1996). Structure of *Mitf* gene is regulated by alternative splicing and extremely complex because of distinct promoters and multiple exons (Hershey & Fisher, 2005). Melanocyte-specific isoform of MITF-M is the most abundant and best-studied MITF isoform (Fuse et al., 1996), whose activity is regulated by cAMP, SOX10, PAX3 and Wnt signalling. Mitf, in turn, governs the transcription of genes involved in melanin synthesis such as tyrosinase (Tyr), tyrosinase-related protein-1 (Tyrp1) and dopachrome tautomerase (DCT or Trp-2) (Bentley et al., 1994; Hemesath et al., 1994). Other numerous melanoma-specific genes have been identified that are involved in various signalling pathways, processing and pigment export.

Among the different lineages derived from neural crest progenitors, excessive activation of Wnt pathway directs differentiation predominantly towards melanocytic lineages (Dorsky et al., 1998). Mitf expression through canonical Wnt pathway is governed the respective proximal elements in the promoter region (Dorsky et al., 2000; Widlund et al., 2002), suggesting the role of Mitf as an alternative co-activator for certain β-catenin-independent targets. Furthermore, Wnt5a has been shown to be essential for melanoma progression as it increases its invasive potential (Weeraratna et al., 2002). Along with above-mentioned pathway, a lot of different signallings are involved in melanoma initiation and progression. Thus, PI3 kinase–AKT pathway is often hyperactive in melanoma (Meier et al., 2005). Hyper-activation of RTK signalling and its mediator MAP kinase pathway (ERK1/2) have been directly linked to melanoma growth-promoting behaviour (Takata et al., 2005). Mutations in *BRAF* gene are the most prevalent somatic genetic events in human melanoma (Davies et al., 2002). It is interesting that in contrast to the majority of cancers, mutations of *TP53* in melanomas are quite low (less than 5%) (Chin, 2003), suggesting that p53 signalling is not relevant for melanoma formation.

Different theories of melanoma progression exist (Schatton et al., 2008). One of them is appearance of melanoma from the progenitor stem cells. Melanocytic stem cells reside within a specialized stem cell niche called the bulge (Fuchs, 2007), which is the source of differentiated melanocytes not only of the hair follicle, but also of the basal epidermis (Nishimura et al., 2002). Melanocytic stem cells are subject to certain differentiation stages characterized by expression of specific markers, finally inhabiting the basal epidermis as well as the hair follicles in human. Temporarily amplifying melanocytes can break away from the niche and migrate to the epidermis, where they further continue to differentiate into pigmented melanocytes. Moreover, some of these transiently amplifying melanocytes can return to the inactive state or even revert to stem cells. Recently, CD20-enriched subpopulation of melanoma cells that grew as nonadherent spheroids in human ES-like medium was identified (Fang

et al., 2005). These CD20+ cells support stem-cell phenotype and could differentiate towards multiple lineages, including melanocytes, adipocytes, osteoblasts, and chondrocytes. However, connection between melanoma stem cells and melanocytic stem cells remains yet to be defined.

1.12 Cadherin switch during normal developmental processes and in tumour progression

Cell adhesion is a very important part of normal cell integrity and homeostasis. Depending on the human tissue type, various classes of adhesion molecules exist. Cadherins, including E- (epithelial), N- (neural), and P- (placental) cadherins, are transmembraneous calcium-dependent glycoproteins. In the process of development, cells have to rapidly adapt to tissue rearrangement and expression of one type of the cadherin is switched to the other, allowing cells to segregate from one another. Patterns of cadherin expression during development and in pathology are dynamically changing and associated not only with the developmental stage or progression of disease but also with the physical location of the cell in the body. Thus, cancer cells use cadherin switching to abolish cell-cell contacts, gain motile phenotype and metastasize (Stemmler, 2008; Wheelock et al., 2008).

In general, E- and N-cadherins are functionally identical with similar protein structure; both form complexes with catenins and act redundantly (Nollet et al., 2000). However, cadherins differ in their selectivity of interaction partners: E-cadherin preferentially interacts with the EGFR, while N-cadherin preferentially interacts with FGFR1 (Fedor-Chaiken et al., 2003; Suyama et al., 2002). In homozygous embryos, N-cadherin is able to substitute E-cadherin function during morula compaction, but incapable to replace E-cadherin during the formation of the blastocyst, indicating to the importance of E-cadherin in trophectoderm formation (Kan et al., 2007). Furthermore, ES cells derived of *E-cadherin* knockout models are incapable of germ layer formation (Larue et al., 1996). Overexpression of E-cadherin in null ES induces epithelia formation, while N-cadherin predominantly stimulates neuroepithelia and cartilage development.

Cadherin switch with the loss of E-cadherin and gain of N-cadherin expression occurs during melanoma development and results in the detachment of melanoma cells from the epidermis and supports their invasion to neighbouring tissues (Li et al., 2002). Among others, PI3K/PTEN signalling is involved in the cadherin switch during melanoma progression (Hao et al., 2012). E-cadherin inactivation may occur due to somatic mutations, promoter hypermethylation, transcriptional and post-translational control of the gene expression (Peinado et al., 2004). Other studies on regulation of E- to N-cadherin switching correlate the extent of this process with the levels of

cytoplasmic β-catenin (Nelson & Nusse, 2004). Sequestering of β-catenin to the nucleus downregulates E-cadherin levels. Moreover, TCF/β-catenin complex binds directly to the promoter of E-cadherin and represses it (Jamora et al., 2003).

One of the important aspects where cadherin switch actively participates is epithelial-mesenchymal transition. During embryonic development, EMT is required for the formation of endodermis, neural crest and craniofacial structures and gastrulation. In cancer, EMT promotes invasion and metastasis and is accompanied with the acquisition of spindle-shape cell morphology, increased migratory capacity, resistance to apoptotic stimuli, absence of expression of epithelial markers and cytokeratins, and increased expression of mesenchymal marker genes (for example, N-cadherin, fibronectin, vimentin) (Zeisberg & Neilson, 2009).

1.13 TAF4: structure, function and regulation

TAF4 is one of the largest and ubiquitously expressed subunits of TFIID complex and is important for maintaining stability and integrity of TFIID (Malkowska et al., 2013). Historically, TAF4 was the first TATA-binding protein associated factor with demonstrated co-activator function. TAF4 has been demonstrated through recent studies to interact with different activators, including Sp1 (Hoey et al., 1993), CREB (Ferreri et al., 1994), N-CoR (Wei et al., 2007), E-box transcription factors (Chen et al., 2013) and c-Jun (Liu et al., 2008). TAF4 transcriptional activation is potentiated by the AF-2 domains of RARα, vitamin D3 and thyroid hormone receptors (Mengus et al., 1997). TAF4 interacts with HP1α and HP1γ, but not HP1β, further complicating its regulatory mechanisms and functions (Vassallo & Tanese, 2002). It is important to note that *TAF4* gene is duplicated in mammals and has an ovarian cell-specific paralogue *TAF4b* (Falender et al., 2005).

The molecular structure of TAF4 is remarkably conserved throughout the evolution. Human TAF4 protein consists of N-terminal metazoan-specific polyglutamine tract region, central co-activator binding TAFH domain (ETO, CRI), and C-terminal histone-fold domain (HFD, CRII) (Wang et al., 2007). TAF4 HFD is conserved from yeast to human and plays a critical structural role in the formation of TFIID. By analogy with histones H2A and H2B, TAF4 interacts with TAF12 through structurally similar HFDs (Werten et al., 2002), (Gnesutta et al., 2013). Formed octamer-like sub-structures support the integration of TAF4 into TFIID complex and its further core promoter region recognition within the chromatin. The length of the DNA sequence that is recognized by the TAF4/TAF12 dimer, is about 70 bp, and is half of the size of that of the nucleosomal DNA. This suggests the formation of hetero-complex structures, similar to the nucleosome complex assembly (Gazit et al., 2009).

TAF4 HFDs are sufficient to nucleate the assembly of holo-TFIID complexes. The yeast version of TAF4 displays the HFD, but possesses neither glutamine-rich amino terminus nor TAFH domain. Structural analysis of HFD of TAF4 revealed an unstructured short sequence located between helixes two and three that is characteristic to TAF4 HFDs only (Thuault et al., 2002). In metazoan, TAF4-TAFH domain is highly conserved across all TAF4 and ETO family proteins (Wang et al., 2007). Structural studies revealed that the α-helical folds of hTAF4-TAFH domain forming a large hydrophobic groove are responsible for protein-protein interactions. Using the phage display screening method, the amino acid sequence that is targeting TAF4-TAFH binding surface was determined as DΨΨζζΨΦ (where Ψ represents V, I, L, or M; ζ represents hydrophilic residues including N, Q, S, or T; and Φ represents V, I, L, F, W, Y, or M) and was found to be present in different transcriptional regulators. Thus, potential TAF4-TAFH binding partners include PBX proteins, important in limb development and hematopoiesis; HCF-1-dependent ZF and LZIP activators participating in chromatin modification and cell proliferation; Mediator subunit MED23, involved in post-translational remodelling of chromatin substrates; and a number of histone deacetylases, demethylases and kinases regulating cell cycle. Different to ETO-TAFH, vertebrate TAF4-TAFH domains exhibit unique packing of helix five, and as a result, present a very flat and extended binding surface. This allows a much broader spectrum of substrate interactions and thus serves as a platform for positive and negative regulation of transcription.

In *Drosophila*, RNAi-mediated depletion of Taf4 affects dramatically TFIID stability, suggesting that TAF4 is one of its key structural subunits (Wright et al., 2006). Moreover, TAFH was identified as a domain targeted by Pygopus that promotes transcription of Wg/Wnt target genes throughout *Drosophila* development (Wright & Tjian, 2009).

In *C. elegans*, depletion of TAF4 is highly destructive; its effects are similar to the RNA Pol II knockout phenotype (Guven-Ozkan et al., 2008). Reversible and controlled repression of TAF4 is one of the mechanisms that regulate cellular development and differentiation. In the early embryo, TAF4 interacts with OMA-1/2, proteins that are necessary for oocyte maturation. This interaction interferes with TAF4 and TAF12 dimer formation and ultimately results in the sequestration of TAF4 to the cytoplasm. Degradation of OMA proteins in cytoplasm releases TAF4, which can now bind TAF12 and the resultant TAF4/TAF12 heterodimers translocate to the nucleus to restore transcription.

In mouse, Taf4 is not essential for fibroblast viability, but its inactivation affects proliferation (Mengus et al., 2005). Notably, *Taf4*$^{-/-}$ fibroblasts contain intact TFIID and do not exhibit cell cycle arrest or apoptotic phenotype. Interestingly, *Taf4* inactivation is accompanied by alterations in cellular morphology, serum-independent autocrine growth and deregulation of more that

1000 genes. These changes in gene expression together with suppression of serum-independent growth are restored by re-expression of protein isoforms containing the CRII domain. *Taf4* depletion leads to high expression of TGFβ, thereby enhancing SMAD signalling by the positive feedback loop. Loss of *Taf4* further induces the expression of matrix metalloproteases, CTGF and OPN, which are important regulators of metastasis, thereby contributing to the oncogenic functions of TAF4. Moreover, Taf4 was demonstrated to act as a co-factor of retinoic acid receptors, and that its CRII domain alone is sufficient to mediate CREB and RAR activities (Fadloun et al., 2008). Ectopic expression of truncated forms of TAF4, containing TAFH but not CRII domains, yielded in accelerated cell growth. In contrast, cells expressing TAF4 CRII alone display slowed rate of growth (Gazit et al., 2009).

Recent studies have focused on the function of TFIID, and TAF4 in particular, in reprogramming of embryonic and somatic stem cells. ChIP sequencing data revealed that Nanog and Oct4 are both recruited to the regulatory region of mouse *Taf4* gene, which acted as ESC-specific enhancer (Chen et al., 2008). In MEFs and adult human fibroblasts, exclusion of TAF4 from the TFIID complex inhibited reprogramming of the somatic cells while TAF4 overexpression facilitated iPSCs formation (Pijnappel et al., 2013). These findings evidence a positive feedback circuit between TAF4 and the pluripotency. Namely, enforced expression of TAF4 activates expression of the pluripotency factors, which in turn enable to maintain high levels of TAF4 expression.

TAF4 has been shown to control cell differentiation. Targeted proteolysis of Taf4 is observed in differentiating mouse F9 embryonal carcinoma cells (Harris & Childs, 2002) and during differentiation of C2C12 myoblasts into myotubes (Perletti et al., 1999; Deato & Tjian, 2008). In contrast, enhanced expression of Taf4 impairs endodermal differentiation with appearance of atypical elongated cAMP-resistant cells, whereas reinforced expression of CRII domain had no effects in this differentiation process in F9 cells (Fadloun et al., 2008). It is interesting to note that enforced expression of TAFH domain alone in F9 cells blocked differentiation towards the early endoderm. These results showed for the first time that regulated degradation of TAF4 is required for differentiation into selected cell lineages.

TAF4 activity is vital for various cellular physiological processes. One of these is the control of epidermal development and homeostasis. Selective loss of *Taf4* in the mouse fetal epidermis results in skin aberrancies, enhanced water loss and early post-natal death, suggesting defective skin barrier function (Fadloun et al., 2007). In adult mouse epidermis, Taf4 participates in normal hair cycle, as *Taf4* deficiency results in fur loss. In addition, *Taf4* inactivation induces epidermal hyperplasia and aberrant differentiation of mouse adult basal keratinocytes. Moreover, *Taf4* inactivation significantly alters cell adhesion, cell communication and induces the expression of markers correlating with

oncogenic transformation. These changes stimulate tumour formation by enhancing malignant transformation. Upon histological analysis, the tumour cells exhibit melanocyte-like phenotype with high expression of the genes involved in melanocyte signalling. However, it is worth noting that a set of genes affected by the loss of T*af4* in mouse keratinocytes is different from that seen in the embryonic fibroblasts or foetal epidermis, suggesting the involvement of different regulatory pathways. In fibroblasts, the TGFβ pathway is activated, whereas in keratinocytes the EGF signalling is enhanced. Other studies connect *Taf4* inactivation in MEFs with the formation of fibrospheres and activated expression of pro-oncogenic Collagen 6A3 (Martianov et al., 2014). The specific mechanism involves repression of Hippo signalling and activation of the Wnt pathway. Interestingly, treatment of MEFs by ATRA restores TAF4-abolished effects.

Thus, implicated in the majority of vital cellular processes, TAF4 as the component of the general transcriptional machinery is a valuable target for controlling cell functions.

THE AIMS OF THIS RESEARCH

The aims of this study were to characterize alternative splicing of human *TAF4* and study the function of protein isoforms generated from alternative transcripts of *TAF4* in cell differentiation and in cancer development.

For that purposes the current tasks were carried out:

1. Characterization of expression patterns of alternatively spliced transcripts of *TAF4* in human cells and tissues.
2. Study the function of TAF4 protein isoforms by using different experimental models.
3. Assess the impacts of *TAF4* ASVs expression on cancer.

MATERIALS AND METHODS

The following methods were used during the study:

- Bioinformatics, sequence alignment, searches against biological databases, and other
- DNA sequencing
- Cultures of primary and immortalized mammalian cells
- *In vitro* differentiation along adipogenic, osteogenic, chondrogenic and neural lineages
- siRNA and DNA transfection of cells
- RNA isolation, reverse transcription
- RT-PCR and RT-qPCR
- Subcellular fractionation
- Preparation of cell lysates and Western blotting
- Flow cytometry
- Immunofluorescence microscopy
- Cytochemical staining
- WST-1 cell proliferation assay
- Cell senescence-associated assay
- Melanin content assay
- Transwell cell migration and invasion assays
- Statistical analysis

RESULTS AND DISCUSSION

1.1 Analysis of alternative splicing regulated *TAF4* expression in human cells and tissues (Publications I–IV)

The previous analysis of the tissue-specific expression of *Taf4* in mice evidenced multiple and complex patterns of alternative splicing (Brunkhorst et al., 2004). In order to describe the role of alternative splicing in transcription of *TAF4*, we designed gene-specific primers to different exons of *TAF4* and performed RT-PCR analysis of different human tissues and cells. As confirmed by sequence analysis of PCR products, a significant number of alternatively spliced *TAF4* mRNAs have preserved ORF and could possess therefore functionally different properties. Depending on the cell type, a different balance of expression between the full-length and the rest of the *TAF4* alternative splice variants was observed. Some of the ASVs of *TAF4* were invariantly expressed across tissues, while others exhibited patterns of cell-type-specific expression.

As identified, the most prone to the alternative splicing are exons of *TAF4* encoding the functional co-activator-binding hTAF4-TAFH domain (Fig. 3). Vertebrate TAF4-TAFH possesses a five-helix structure that is responsible for protein-protein interactions and recognizes a hydrophobic DΨΨζΨΦ motif (similar to LxxLL) of TAF4 interaction partners (Wang et al., 2007). Thus, cell-specific splicing, targeting the structural integrity of hTAF4-TAFH, presumably affects its interaction properties with consequences on cell function. To study the role of specific splice variants in the cell context, we affected *TAF4* alternative splicing by siRNA, targeting the sequences encoding the hTAF4-TAFH domain. Our data show that in response to TAFH RNAi treatments, cells start to express alternatively spliced variants of *TAF4*, such as *TAF4_v2, TAF4_v4* or *TAF4_v5* mRNAs that encode protein isoforms with only two helixes out of five preserved from the canonical structure of hTAF4-TAFH. Alternative splicing of exons VI and VII alters the flat and wide binding surface of hTAF4-TAFH domain, making it more similar to that of the ETO-TAFH domain. ETO-TAFH-dependent interactions with LxxLL-carrying proteins, including LZIP, E-proteins, nuclear hormone receptors, and subunits of Mediator complex, have been suggested to affect the whole PIC composition and activity (Plevin et al., 2005). Thus, splicing of exons encoding the hTAF4-TAFH domain ultimately contribute to the changes in target gene expression, perhaps allowing the fine-tuning of the transcriptional response to activators that is important during development.

As mentioned above, TAF4 is one of the structural components of TFIID complex and affects its stability. Therefore, alternative splicing of *TAF4* has the most severe consequences on the integrity and functional activity of TFIID, as well as that of PIC as a whole. Consistent with this, some of its alternative

transcripts generate alternative protein isoforms with functions that differ from canonical TAF4 functions; otherwise, certain alternative mRNAs of *TAF4* could function as long non-coding RNAs. The latter remains to be proven.

Analyses of *TAF4* alternative splicing of cells with differentiation potential, such as mesenchymal stem cells, dermal fibroblasts, and neural progenitor cells, revealed the dominant expression of ASVs with intact hTAF4-TAFH domain. However, on the protein level, the canonical form of TAF4 corresponding to *TAF4_v1* is expressed at relatively low levels.

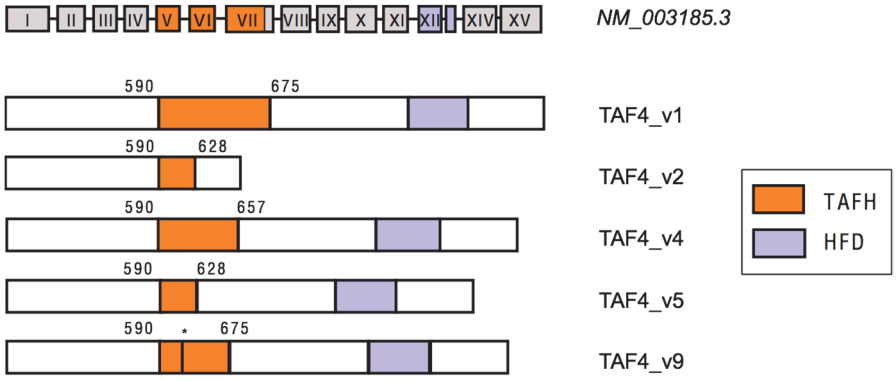

Figure 3. Alternative splicing of human *TAF4* (*NM_003185.3*) leads to a variety of protein isoforms. *TAF4* gene structure and major TAF4 isoforms with deletions in the co-activator-binding hTAF4–TAFH domain are outlined. Some isoforms of TAF4, for example TAF4 ASV_v2, differ in their *C*-terminus. Functionally important TAFH (hTAF4–TAFH, orange) and HFD (Histone-fold, blue) domains are highlighted. Boundaries of hTAF4–TAFH domain are shown by amino acid residue numbers above the structures and correspond to their position in the canonical form of TAF4, where the * symbol represents deletion of the amino acids 628–658 in the TAFH domain.

1.2 The balanced expression of *TAF4* ASVs affects cellular differentiation (Publications I, II, and IV)

Analysis of *TAF4* ASVs expression of hMSCs differentiated into adipocytes, osteoblasts, and chondrocytes detected high levels of expression of mRNAs with skipped exons encoding hTAF4-TAFH domain. Notably, the most abundantly upon differentiation expressed mRNA was *TAF4_v2*, with no intact hTAF4-TAFH domain. These data indicated that alternative splicing of *TAF4* is involved in cell differentiation.

To elucidate the role of *TAF4* alternative splicing on cell differentiation, we manipulated with the balance of *TAF4* ASVs by using RNAi or transient overexpression approaches. We designed two different siRNAs targeting different exons encoding hTAF4-TAFH. Our data demonstrated that TAFH RNAi was effective and led to the appearance of new ASVs in hMSCs, NHNPs, and dermal fibroblasts. These changes were accompanied with downregulation of canonical *TAF4_v1* and upregulation of alternative transcripts encoding partially or entirely lost hTAF4-TAFH. Directed differentiation of TAFH siRNA-treated cells revealed lineage-specific effects of changed *TAF4* ASV patterns on cell differentiation (Kazantseva et al., 2013). The progression and efficiency of each differentiation programme were checked by dynamic analyses of respective molecular marker genes on RNA and protein levels together with specific histological staining analysis. Namely, in human adipose-derived MSCs, silencing of canonical TAF4 activity contributed to the repression of adipogenic and osteogenic programmes, while promoted chondrogenesis (Kazantseva et al., 2013). Similarly, TAFH RNAi induced expression of ASVs and stimulated neural (glial and neuronal) differentiation of NHNPs (Kazantseva et al., 2014). Adding support to the hypothesis that sustained expression of TAF4 impairs cell differentiation, TAFH RNAi induced spontaneous differentiation of facial dermal fibroblasts into melanocyte-like cells (Kazantseva et al., manuscript). Phenotypic features of the melanocyte-like conversion of TAF4-deficient fibroblasts included darkening of the cell pellets due to melanin secretion and high levels of expression of melanogenic-specific transcription factor MITF and its target genes *TYR* and *TYRP1*. Interestingly, deregulation of hTAF4-TAFH domain by TAFH-specific siRNAs in human dermal fibroblasts is comparable with total TAF4 disruption by a mixed pool of three distinctive siRNAs designed against different from hTAF4-TAFH parts of the gene (data not shown). This observation emphasizes the importance of the hTAF4-TAFH functional domain for the entire protein activity.

Next, we asked whether high expression of canonical TAF4 has opposite effects on cell differentiation. Indeed, enforced expression of full-length *TAF4* contributed to increased levels of expression of *SOX2*, an important marker of the neural stem-cell state, and decreased expression of specific glial and neuronal genes.

Altogether, these data evidence that *TAF4* ASVs can switch the balance between proliferation and differentiation. Suppression of hTAF4-TAFH activity accelerates differentiation of stem and progenitor cells, while predominant expression of the canonical form of TAF4 keeps stem-like cells in an undifferentiated state.

1.3 Silencing of hTAF4-TAFH activity results in activation of p53 and non-canonical WNT signalling (Publications I and II)

Amongst detected outcomes, the influence of *TAF4* siRNAs on hMSCs was decreased cell proliferation and changed cell cycle. A 6-h treatment of hMSCs and NHNPs with TAFH siRNAs already resulted in the upregulation of *CDKN1A* and *CDK2* levels. Western blot analysis revealed that opposite changes in the expression of *TAF4_v1* and cell cycle regulators *CDKN1A* and *CDK2* mRNAs led to a marked decrease in the expression of the canonical TAF4 protein and significantly increased CDKN1A levels. The exact mechanisms are currently unclear, although our initial findings revealed the involvement of p53 signalling pathways, as we detected accumulation of *TP53* expression and hyperphoshporylated $TP53^{Ser15}$ in all cells in response to TAFH RNAi. Recently, TAF4 was detected in a TP53-binding site on the *CDKN1A/P21* promoter and this interaction was enhanced in response to UV irradiation (Li et al., 2007). Interestingly, in hTAF4-TAFH-depleted MSCs, increased levels of TP53 induced cell cycle arrest without any signs of apoptosis or cell senescence (Kazantseva et al., 2013).

Additionally, our data show switching from canonical to non-canonical WNT pathways in response to TAFH RNAi in hMSCs and NHNPs (Kazantseva et al., 2013). Previous works demonstrated that WNT signalling promotes activation of WNT target genes by targeting Pygopus-TAFH interactions in *Drosophila* (Wright & Tjian, 2009; Marr, 2009). Our data show that TAFH-specific RNAi results in downregulation of β-catenin expression and upregulation of *DKK1* and *WNT5A*, indicating to the enforced switching between canonical and non-canonical WNT pathway activation. It is interesting to note that other studies have found that suppression of canonical WNT signalling supports chondrogenesis, contributes to cell migration (Topol et al., 2003), and affects bone formation and limb development (Niehrs, 2006). In neural progenitors, both canonical and non-canonical WNT signalling has been found to control neuronal differentiation in a developmental stage-specific manner (Montcouquiol et al., 2006; Davis et al., 2008; Munji et al., 2011). Thus, the switch from canonical to non-canonical WNT signalling could be one of the regulative mechanisms in TAF4 activity-deficient progenitor cells. To further confirm the interplay of TP53 and WNT signalling that was observed in TAFH siRNA treated cells, previous studies have shown that TP53 and its target *miR-34* suppress canonical WNT signalling (Kim et al., 2011). Next, a

genome-wide screening study has revealed that the WNT signalling pathway is one of the major targets of TP53 activity (Lee et al., 2010). Furthermore, p53 signalling appears also to play a role in differentiation of TAF4-deficient mesenchymal stem cells: several recent studies link TP53 activity to cell differentiation (Armstrong et al., 1995), in addition to its well-established role in apoptosis.

Overall, our findings are in good agreement with the previous studies and strengthen the notion that *TAF4* ASVs encoding TAF4 isoforms with altered hTAF4-TAFH activity control cell differentiation through synergistic activation of TP53 and WNT pathways, although the detailed mechanisms have yet to be established.

1.4 Imbalanced expression of *TAF4* ASVs affects migration of facial dermal fibroblasts and melanoma cells (Publication IV)

Differentiation during development is often accompanied by increased cell motility that is necessary for cell homing. Therefore, we specifically analysed the effects of TAFH RNAi on migration of fibroblasts. We observed that inhibition of TAF4 activity strongly enhances cell migration. Increased cellular motility was accompanied by events reminiscent of the epithelial-to-mesenchymal transition, as *TAF4* silencing inversely correlated with cadherin switch: downregulation of TAF4 in the dermal fibroblasts suppressed E-cadherin and supported N-cadherin expression. It is known from other data that cadherin switch contributes to cancer progression and emergence of cells with stem-like characteristics (Mani et al., 2008).

In association with these findings, we used TAFH RNAi approach in melanoma cells. As a result, TAFH siRNA treatments suppressed the expression of *TAF4_v1* and altered the patterns of expression of *TAF4* ASVs, thereby shifting the balance of *TAF4* expression towards alternative transcripts encoding dysfunctional hTAF4-TAFH domain. Furthermore, TAFH RNAi stimulated the invasion of melanoma cells and, similarly to TAFH RNAi-treated fibroblasts, demonstrated the downregulation of E-cadherin and upregulation of N-cadherin expression that was followed by increased expression of *MMP3* and decreased expression of epithelial marker *KRT14*. All these molecular alterations provide the cells with a potential to segregate from the tissue (neighbourhood) and metastasize. In contrast, enforced expression of canonical TAF4 in melanomas shifted expression of the respective marker genes towards reduced invasiveness compared with the control treatments. Thus, alteration in hTAF4-TAFH activity significantly affects the migration potential of melanoma cells and also affects their ability to invade through the extracellular matrix components, thereby controlling the tumour initiation and progression.

1.5 Cancer stem cell properties of melanoma are controlled by *TAF4* ASVs (Publication IV)

Cells that are prone to multi-lineage differentiation, such as fibroblasts and mesenchymal stem cells, express canonical TAF4 at low levels compared with the remarkable expression of ASVs encoding TAF4 isoforms. At the same time, high expression of full-length TAF4 keeps cells in the pluripotent state (Pijnappel et al., 2013). In contrast, cancer cells such as melanomas express both canonical and other isoforms of TAF4 at high levels, thereby raising the question of the differentiation potential of tumour cells. Overall, highly plastic and heterogeneous melanoma cells fit well to the model of being cancer stem cell derived. Melanoma cells express pluripotent and differentiation-associated genes and differentiate into a wide range of cell types (Na et al., 2009). On the other hand, TAF4 as a pluripotency-associated stem-cell factor has been shown to facilitate the reprogramming of fibroblasts into the pluripotent stem cells (Pijnappel et al., 2013). The notion that fully differentiated cells have low levels of TAF4 activity is further supported by the findings that either canonical TAF4 is barely detected in differentiated cells or its functional activity is lost due to the directed proteolysis (Deato & Tjian, 2007; Goodrich & Tjian, 2010; Perletti et al., 2001).

Our data revealed that shifting patterns of *TAF4* ASV expression either towards the full-length mRNAs or towards transcripts with skipped exons encoding hTAF4-TAFH might govern self-renewal and differentiation mechanisms also in melanomas. By analogy with human neural progenitor cells (Kazantseva et al., 2014) and MSCs (Kazantseva et al., 2013), downregulation of TAF4 activity promotes melanoma cells to differentiate. Our results showed that TAFH RNAi treatment of melanoma cells resulted in downregulation of *KLF4*, *OCT4,* and *NANOG* expression, and simultaneous downregulation of the expression of melanocyte-specific genes such as *MITF* and *TYR.* Furthermore, expression of genes that are characteristic for differentiation towards chondrogenic, adipogenic, and neural lineages was increased in hTAF4-TAFH -silenced melanoma cells when compared to cells treated with control siRNAs. In contrast, in case of high TAF4 activity, melanoma cells activate multipotency programmes similarly to non-malignant precursor cells (Kazantseva et al., 2014; Pijnappel et al., 2013). Thus, upon enforced expression of canonical TAF4, the increased expression of stem cell markers such as *KLF4*, *NANOG,* and *OCT4* was observed in melanoma cells, evidently contributing to their multipotent characteristics.

Altogether, our data using *TAF4* gain- and loss-of function studies allow us to conclude that melanomas have properties of stem cells and exhibit multi-lineage differentiation potential (Kazantseva et al., manuscript).

1.6 Disturbances in *TAF4* alternative splicing as a cause of melanoma initiation and progression (Publication IV)

Primary melanomas and cultured melanoma cells share similar patterns of expression with high levels of expression of both, full-length *TAF4_v1* and *TAF4* ASVs with almost completely (such as *TAF4_v2*) or partially skipped (*TAF4_v4* and *TAF4_v6*) exons encoding for hTAF4-TAFH domain. In comparison, fibroblasts possess similar patterns of *TAF4* ASV expression as mature melanocytes. Analogous findings were received from protein analysis data confirming that different to the multipotent stem-like cells, such as hMSCs, NHNPs, and fibroblasts that express low levels of canonical TAF4, melanoma cells co-express the canonical form of TAF4 protein together with alternative isoforms with incomplete hTAF4-TAFH domains at equal or higher levels. Thus, *TAF4* alternative splicing is distinctly regulated in melanoma cells by combining splicing patterns of multipotent and differentiated cells. In our understanding, impairments in the alternative splicing patterns of *TAF4* by external or environmental stimuli could affect cell fate during normal cellular homeostasis and contribute to carcinogenic transformation.

Genetic and biochemical studies have established the role of TAF4 in carcinogenesis by targeted inactivation of *Taf4* in mouse basal keratinocytes (Fadloun et al., 2007). Absence of *Taf4* led to ectopic expression of melanocyte-specific melan-A, melanoma-associated antigen 9 (MAGEA9), tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT) in the adult mouse epidermis, apparently affecting melanocyte-specific signalling pathways and ultimately fostering development of melanomas. It is interesting to note that the loss of *Taf4* in the mouse epidermis altered skin morphology and histology (Fadloun et al., 2007). In our work, in response to TAFH siRNA-treatments of facial dermal fibroblasts, we detected the appearance of melanocyte-like cells with enhanced migration potential. Additionally, unbalanced and ectopic expression of *TAF4* ASVs led to heightened invasion of melanoma in transwell migration assay. All these data indicated that alternative splicing of *TAF4* is apparently driving stem cell properties in melanoma cells, thereby proposing it to be the first or a key step in the progression of malignant transformation of the dermis.

1.7 Concluding remarks (Publication III)

Herein we show that regulation of TAF4 activity by alternative splicing is an example of the fast adaptation of the cellular machinery to vital needs. In differentiated cells, the balance of *TAF4* ASV expression is shifted towards generation of isoforms with impaired hTAF4-TAF4 domain, while dominant expression of the canonical TAF4 form is necessary for maintenance of the pluripotent state of the cell. Instead, drastic alterations of *TAF4* ASV

equilibrium could initiate tumorigenesis. Thus, melanoma cells expressing both canonical and alternative *TAF4* transcripts at equal levels exhibit both, multipotency and inclination to differentiate. By affecting this balance, it is possible to manage these processes more effectively. Thus, normal and cancer cells control their fate by using one gene in its different manifestations, e.g. changing levels of *TAF4* ASV expression.

Targeting the self-renewal and differentiation potential of stem cells for clinical use is worthless if the migration of cells to target tissues cannot be appropriately controlled. Migration of stem cells to different organs and target niches requires active guidance, a process termed homing. Homing is necessary for tissue transplantation and for seeding stem progenitors during development. Being better understood and studied for hematopoietic stem cells, it is also applicable to mesenchymal and cancer stem cells (Pittenger & Martin, 2004). Furthermore, considering that cancer is often a stem-cell-retaining disease, it is important to understand the different features of homing and migration of cancer and normal stem cells. Our data demonstrate that reduced expression of full-length *TAF4* by RNAi leads to the enhanced motility of normal dermal fibroblasts and malignant melanoma cells (Kazantseva et al., manuscript). In contrast, transient overexpression of canonical TAF4 diminishes the invasion potential of the melanoma cells. Observed changes are accompanied by molecular switches in the cell matrix.

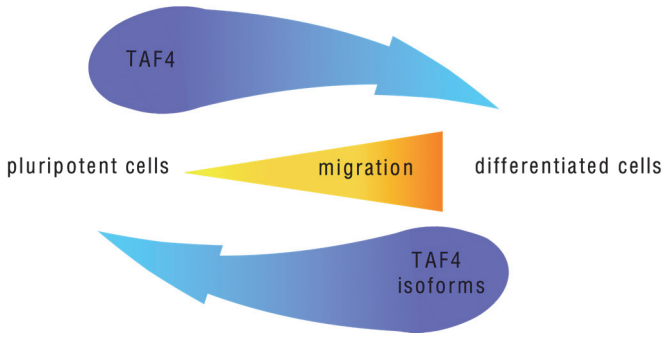

Figure 4. A model describing the cross-talk between canonical TAF4 and its alternative isoforms in pluripotent (stem cells) and differentiated cells, and their relation to migration. High levels of canonical TAF4 (*blue arrow from left to right*) are a characteristic to normal stem cells and cancer cells. In contrast, differentiated cells maintain high expression of TAF4 isoforms with deletions in hTAF4-TAFH domain (*blue arrow from right to left*). Functional balance between canonical and alternative isoforms of TAF4 is crucial for cell migration. Consistent with this, cells with low expression of canonical TAF4 are prone to migration contrasting with low motility of cells with high levels of TAF4 activity.

Altogether, our studies identify TAF4 as a critical modulator of cell differentiation and metastatic spread of cancer (Fig. 4). As a whole, the regulated expression of canonical and other TAF4 isoforms reveals a remarkable conservation in different cell systems, and supports cell state transitions (between pluripotency and differentiation) and migration. Loss of hTAF4-TAFH activity by regulated alternative splicing affects the cell homeostasis of normal and cancer cells. By affecting the equilibrium of *TAF4* alternative mRNAs encoding functionally different protein isoforms, it is possible to direct cell status either towards pluripotency or differentiation. This finding is reinforced by the observation that the motility of normal and malignant cells and the expression of full-length *TAF4* are inversely correlated, thereby combining the concepts of cell migration in development and disease progression.

CONCLUSIONS

1. Patterns of *TAF4* ASV expression in human cells and tissues are complex and diverse. Human *TAF4* ASVs are expressed ubiquitously but not with equal distribution across tissues. The part of *TAF4* gene most prone to the alternative splicing is encoding its co-activator-binding hTAF4-TAFH domain.
2. Silencing of hTAF4-TAFH activity in hMSCs suppresses proliferation, and has no effects on apoptosis or cell senescence.
3. The predominant expression of *TAF4* transcripts encoding isoforms with impaired hTAF4-TAFH domain a) accelerates differentiation towards chondrogenic lineages but inhibits adipo- and osteogenesis of human MSCs; b) facilitates neural differentiation of neural progenitor cells; and c) leads to spontaneous conversion of facial dermal fibroblasts into melanocyte-like cells.
4. Downregulation of the canonical TAF4 activity at the expense of that of alternative TAF4 isoforms enhances the motility of human dermal fibroblasts and melanoma cells. Conversely, cells with increased expression of canonical TAF4 possess reduced migration and invasion potential.
5. TAF4-dependent signalling mechanisms involve switching between the canonical and non-canonical WNT pathways, TP53 signalling activation, and shifting in E- and N-cadherin expression.
6. The balance between the expression of the full-length and ASVs of *TAF4* controls the pluripotency or differentiation status of the cells. Cells with high levels of canonical TAF4 are predominantly pluripotent, while preferable expression of non-canonical isoforms of TAF4 directs cells to differentiate.
7. Melanoma cells express canonical and alternative *TAF4* transcripts at equal levels, thereby possessing both, multipotency and differentiation potential in their arsenal. By shifting this equilibrium, it is possible to get melanoma populations with high multipotent properties or direct cells to differentiate.

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SUMMARY

Development is a highly controlled process of cell proliferation and differentiation driven by mechanisms of dynamic gene regulation. For establishing cell- and tissue-specific transcriptional programmes, the function of distinct DNA-binding factors has been analyzed in different cell and animal models. However, much less is known about the role of "general transcription machinery" during normal development and in pathologies. Together with transcription, alternative splicing is a key regulatory mechanism for generating proteomic complexity and promoting phenotypic diversity of eukaryotes.

In this work, we focus on the role of TATA-box protein associated factor 4 (TAF4) and its functional isoforms generated by alternative splicing. The aims of the thesis were (i) to characterize the patterns of alternative splicing of *TAF4* in various human cells and across tissues; (ii) to study the cellular functions of TAF4 isoforms, and (iii) to assess the impact of differential splicing patterns of *TAF4* on cancer progression.

The current study demonstrates that different human cells and tissues have distinct alternative splicing patterns of *TAF4*. Some alternative transcripts are abundant, while others are expressed at low level and in cell-type specific manner. Interestingly, the most prone to alternative splicing regulation is the co-activator-binding hTAF4-TAFH domain. Affecting splicing of exons encoding hTAF4-TAFH domain, the TAF4 activity drastically changes. Applying RNA interference (RNAi) approach, suppression of hTAF4-TAFH function enhanced chondrogenic, but not adipo- and osteogenic differentiation of mesenchymal stem cells (MSCs). In addition, hTAF4-TAFH RNAi resulted in accelerated differentiation of neural progenitor cells and spontaneous melanocyte-like differentiation of facial dermal fibroblasts. Conversely, transient overexpression of canonical TAF4 in neural progenitor cells yielded in decreased expression of neural-specific genes and increased expression of major transcription factors regulating pluripotency. Along with this, melanoma cells express complex patterns of *TAF4* mRNAs. Shifting of these patterns by hTAF4-TAFH RNAi resulted in dominant expression of hTAF4-TAFH-impaired ASVs and effective initiation of neuronal and chondrogenic differentiation programs in melanoma cells. In contrast, high levels of canonical TAF4 activity in melanoma cells increased the expression of the core pluripotency factors. Finally, changes in the expression of *TAF4* alternative splice variants (ASVs) had severe consequences on cell motility as assessed by using transwell migration assays. Fibroblasts and melanoma cells with enforced predominant expression of full-length *TAF4* have decreased motility, while impairment of hTAF4-TAFH function promoted cellular migration via E-/N-cadherin switching. Altogether, these findings suggested the key role of *TAF4* ASVs in the malignant transformation of dermis.

In conclusion, the work done in this thesis provides novel insights to the role of alternative splicing of *TAF4* in multipotency maintenance and cancer progression. These results provide new prospect for cellular therapies and could help in searching of new effective anti-cancer strategies.

KOKKUVÕTE

Organismi areng on rakkude jagunemisel ja diferentseerumisel põhinev hästi kontrollitud protsess, mille aluseks on geeni regulatsioonimehanismide dünaamika. Raku- ja koespetsiifilise transkriptsiooni mõistmiseks on põhjalikult uuritud erinevate DNA-ga seonduvate transkriptsiooni faktorite aktiivsust nii raku- kui loomkatsetes. Ometi on oluliselt vähem teada "üldise transkriptsioonimasinavärgi" rollist normaalses arengus ja haiguse patogeneesis. Koos transkriptsiooniga omab alternatiivne splaising võtmerolli eükarüootses geeniregulatsioonis kindlustades rakkude fenotüübiliseks mitmekesisuseks vajaliku proteoomi komplekssuse.

Käesolevas töös keskendusime *TATA-box*- elemendiga seonduva valguga assotseeritud faktori 4 (TAF4) uurimisele ja selle alternatiivsest splaisingust tulenevate isovormide funktsiooni kirjeldamisele. Doktoritöö eesmärkideks oli (i) iseloomustada *TAF4* alternatiivse splaisingu mustreid erinevates inimese rakutüüpides ja kudedes; (ii) uurida TAF4 isovormide rakulisi funktsioone, ja (iii) hinnata *TAF4* erinevate splaisingumustrite mõju vähi tekkele ja progressioonile.

Antud uurimus näitab, et erinevad inimese rakud ja koed omavad erinevaid *TAF4* splaisingumustreid. Samal ajal kui mõned alternatiivsed transkriptid on ulatuslikult ekspresseeritud, on teised madalal tasemel või rakutüübi-spetsiifiliselt avaldunud. Huvitaval kombel on alternatiivse splaisingu regulatsioonist kõige enim mõjutatud ko-aktivaatoriga seonduv hTAF4-TAFH domään. Mõjutades hTAF4-TAFH domääni kodeerivate eksonite splaisingut muutub järsult TAF4 aktiivsus. Rakendades RNA interferentsi (RNAi) meetodit suurendas hTAF4-TAFH funktsiooni mahasurumine mesenhümaalsete tüvirakkude diferentseerumist kõhre-, kuid mitte rasv- ja luukoe suunas. Lisaks põhjustas hTAF4-TAFH RNAi neuraalsete tüvirakkude varast diferentseerumist ning näo piirkonna fibroblastide spontaanset diferentseerumist melanotsüüdi-sarnasteks rakkudeks. Vastupidiselt, kanoonilise TAF4 kõrgendatud tase neuraalsetes tüvirakkudes viis neuronspetsiifiliste geenide ekspressiooni vähenemisele, samas tõstes peamiste pluripotentsust reguleerivate transkriptsioonifaktorite taset. *TAF4* mRNAde mustri muutmine hTAF4-TAFH RNAi abil soodustas melanoomi rakkudes efektiivset neuraalset ja kõhrelist diferentseerumist. Samal ajal, kanoonilise TAF4 aktiivsuse tõus tõi kaasa melanoomi rakkudes peamiste pluripotentsuse faktorite ekspressiooni tasemetes olulise tõusu. Lisaks olid muutustel *TAF4* ASV ekspressioonis tõsised tagajärjed rakkude liikuvusele, mis tuvastati kasutades *transwell* migratsiooni meetodit. Eelistatult kanoonilist TAF4 isovormi ekspresseerivad fibroblastid ja melanotsüüdid on vähese liikuvusega, samal ajal kui hTAF4-TAFH aktiivsuse vaigistamine soodustas E-/N-kadheriinide lülitusmehanismi industeerimise abil olulist tõusu rakkude liikuvuses. Kokkuvõtvalt võib antud tulemuste põhjal

järeldada, et *TAF4* ASV-d omavad üliolulist rolli naharakkude haiguslikes protsessides.

Kokkuvõtteks, käesoleva doktoritöö raames tehtud uurimus annab uudse käsitluse *TAF4* alternatiivse splaisingu rollile rakkude multipotentsuse hoidmisel ja vähi tekkes. Antud tulemused pakuvad uusi väljavaateid rakuteraapilisteks rakendusteks ja võivad kaasa aidata uute efektiivsete vähivastaste ravimite arendamisel.

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Alternative Splicing Targeting the hTAF4-TAFH Domain of TAF4 Represses Proliferation and Accelerates Chondrogenic Differentiation of Human Mesenchymal Stem Cells

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Abstract

Transcription factor IID (TFIID) activity can be regulated by cellular signals to specifically alter transcription of particular subsets of genes. Alternative splicing of TFIID subunits is often the result of external stimulation of upstream signaling pathways. We studied tissue distribution and cellular expression of different splice variants of TFIID subunit *TAF4* mRNA and biochemical properties of its isoforms in human mesenchymal stem cells (hMSCs) to reveal the role of different isoforms of TAF4 in the regulation of proliferation and differentiation. Expression of *TAF4* transcripts with exons VI or VII deleted, which results in a structurally modified hTAF4-TAFH domain, increases during early differentiation of hMSCs into osteoblasts, adipocytes and chondrocytes. Functional analysis data reveals that TAF4 isoforms with the deleted hTAF4-TAFH domain repress proliferation of hMSCs and preferentially promote chondrogenic differentiation at the expense of other developmental pathways. This study also provides initial data showing possible cross-talks between TAF4 and TP53 activity and switching between canonical and non-canonical WNT signaling in the processes of proliferation and differentiation of hMSCs. We propose that TAF4 isoforms generated by the alternative splicing participate in the conversion of the cellular transcriptional programs from the maintenance of stem cell state to differentiation, particularly differentiation along the chondrogenic pathway.

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Introduction

Transcription initiation by RNA polymerase II requires assembly of general transcription factors (GTFs) to form a functional preinitiation complex (PIC). Recent data shows that the composition of the general transcriptional machinery is not static but spatio-temporally regulated during development of different tissues [1]. Genetic, functional and biochemical studies using different cell types and model organisms have revealed the existence of the alternative initiation complexes [2].

Transcription factor complex TFIID is one of the main components of the general transcriptional machinery. It consists of TATA binding protein (TBP) and up to 14 TBP-associated factors (TAFs) [3]. TFIID is essential for promoter recognition and interactions with transactivators [4]. Most recently, it was established that the human TFIID core complex contains two copies each of TAF4, TAF5, TAF6, TAF9 and TAF12 [5]. TBP and TAFs are highly regulated [6,7], whereas modified forms of TFIID selectively act on specific transcriptional networks. For example, TAF4b in cooperation with c-Jun drives tissue-specific programs of gene expression [8], whereas TAF10 is essential for the expression of a subset of genes required for cell cycle progression [9]. The loss of a specific TAF function always affects a defined subset of genes [10] indicating that each TAF has a different and distinct role in transcription of certain but not all genes.

TAF4 plays a critical role in maintaining TFIID complex structural stability [11]. Metazoan TAF4 protein has conserved the N-terminal glutamine-rich domain followed by the co-activator TAF4-homology ETO-TAFH/CRI/NHR1 (TAFH) domain in the central part of the protein and C-terminal histone-like (CRII) domain [12]. The four glutamine-rich domains of human TAF4 have been shown to mediate interactions with activators CREB [13] and Sp1 [14–16]. The interactions of TAFH domain with N-CoR and its repressive activity on transcription through its interaction with E-proteins have been reported [17]. However, the human TAF4 TAFH domain (hTAF4-TAFH) represents a five-helix structure that is characteristic to vertebrates only and therefore has a distinct although related interaction specificity from that of the other TAFH domains [18]. The role of HDACs, methyltransferases, and the PBX family of transcription factors as interaction partners of hTAF4-TAFH has been postulated [18]. The importance of TAFH domain in WNT signaling in *Drosophila* has been

established [19], suggesting the role of hTAF4-TAFH in development, cell fate determination and differentiation.

Structural and functional diversity of cell-specific GTFs and complex molecular mechanisms regulating their activity during development and differentiation are well described. Data on the impact of individual TFIID subunits on cell differentiation are controversial [20–22]. In *C. elegans*, TAF4 in cooperation with zinc finger proteins OMA-1/2 drives global repression of transcription and regulates oogenesis [23]. Undifferentiated embryonic stem cells express only a subset of canonical TAFs, lacking TAF4 [24]. Regulated TAF4 degradation has been reported to be essential for male germ-cell differentiation [25]. TAF4b, a TAF4 paralog, is required for oocyte development [26] and participates in the proper maintenance of spermatogenesis in the mammalian testis [27]. No data on TAF4 developmental expression patterns and the functional effects of directed silencing and deregulation of the hTFA4-TAFH domain have been available up to now.

During developmental signaling, alternative splicing resulting in distinct protein isoforms with specific biochemical properties are a prevalent mechanism in modulating the function of many transcription factors. Alternatively spliced mRNAs are identified for all TAF subunits [28]. Nevertheless, only a few studies have addressed the role of TAF protein isoforms in a cell and tissue specific context. In humans, TAF6δ isoform, in opposed to major TAF6α isoform, triggers apoptosis [7]. In *Drosophila*, two isoforms, TAF1-1 and TAF1-2, differ in their DNA binding activities and contribution to gene-specific transcription [29]. Currently, five alternatively spliced murine *Taf4* mRNAs have been described [30]. These splice variants encode protein isoforms that differ in the structure of their TAFH and CRII domains and affinity for different nuclear receptors. Some are expressed in a cell-type specific manner and exhibit dominant negative effects on nuclear receptor-mediated TAF4 transcription.

Here we describe functional consequences of alternative splicing of TAF4 affecting the integrity of the hTAF4-TAFH domain on human mesenchymal stem cell proliferation and differentiation. Our data suggests that alternative splicing of *TAF4* is one of the key processes influencing stem cell differentiation and reprogramming.

Materials and Methods

Ethics Statement

Experiments using human material were approved by the Ethics Committee of the National Institute for Health Development, Tallinn, Estonia (Approval No 2234 from Dec 09, 2010) and written informed consent was obtained from all participants.

Cell culture

Human MSCs were obtained from freshly isolated subcutaneous adipose tissue as previously reported [31] and published by Kauts et al. [32]. Description of the donors and the use of human cells in different studies are provided in Table S1. The cells were expanded in a medium consisting of low glucose Dulbecco's modified Eagle's medium with glutamine (DMEM-LG) (PAA Laboratories, Austria) supplemented with 1% penicillin/streptomycin (PAA Laboratories) and 10% fetal bovine serum (FBS) (PAA Laboratories) in a humidified atmosphere at 37°C and 5% CO_2. The first plating of the cells after the isolation was designated as passage 0 (P0), and each splitting of confluent cells was considered as the next passage. The cells from passages between P2 and P5 were used in the functional assays in the present study.

siRNA transfection

Small interfering RNAs (siRNAs) targeting exons V and VI of *TAF4* were designed as 5′-GGUUAUACCGAGAACUUAA-dTdT-3′ and 5′- CAGCUAAUGUGAAAGAGCU-dTdT-3′ respectively. *Silencer*® Select *TAF4* siRNAs and Negative Control #2 synthetic scrambled siRNAs were purchased from Ambion, Invitrogen (UK). hMSCs were trypsinized 24 h before transfection and treated with 50 nM of each siRNA using Lipofectamin RNAiMAX reagent (Invitrogen, UK) according to the manufacturer's protocol. Normal growth medium was added 24 h after transfection and changed every three days. All experiments were done using siRNAs targeting exons V and VI of *TAF4*, whereas results generated using siRNAs targeting exon VI of *TAF4* were used for data presentation.

Differentiation Procedures

Approximately 90% confluent hMSCs at passages P2 to P5 were transfected with *TAF4* or control siRNAs and 24 h later treated with adipogenic, chondrogenic or osteogenic type of differentiation media for up to 7 days. Adipogenic differentiation medium consisted of DMEM/F-12 (Gibco, Invitrogen, UK) supplemented with 5% heat-inactivated FBS, 10 µg/ml insulin (Sigma, USA), 0.5 mM IBMX (Sigma, USA), 0.1 mM indomethacin (Sigma, USA) and 1 µM dexamethasone (Sigma, USA). Osteogenic differentiation medium consisted of DMEM-F12 supplemented with 5% heat-inactivated FBS, 10 nM BMP6 (PeproTech, USA), 10 nM dexamethasone, 10 mM and β-glycerolphosphate (Sigma, USA). Chondrogenic differentiation medium consisted of DMEM-High Glucose (PAA Laboratories) supplemented with 10 nM TGF-β1 (PeproTech, USA), 0,1 µM dexamethasone, 1 mM ascorbic acid-2-phosphate (Sigma, USA), 1 mM sodium pyruvate (Gibco, Invitrogen, UK) and 1% insulin-transferrin-selenium-X (Gibco, Invitrogen, UK). Lipid-rich vacuoles were visualized using Oil-Red-O (Sigma, USA) staining performed as described [33]. Osteogenic differentiation was assessed using alkaline phosphatase substrate NBT/BCIP (Pierce Biotechnology Rockford, IL, USA) as described [34]. Chondrogenic differentiation was assessed by immunofluorescence staining.

Immunofluorescence

hMSCs were grown on 22-mm^2 glass slides to about 70% confluency, treated with control or *TAF4* siRNAs and induced with differentiation supporting media for chondrogenic differentiation for 5 days. Cells were washed once with 1xPBS, fixed using 4% paraformaldehyde (Scharlau, Germany) in 1xPBS for 20 min at RT, washed 3 times with 1xPBS and blocked in 1xTBS containing 0.05% Tween20 (TBS-T) and 2% of bovine serum albumin (BSA) for 2 h at RT. Primary antibodies against COL2A1 (Millipore, MAB1330, 1:100) and SOX9 (Millipore, AB5535, 1:500) were diluted in 1xTBS containing 0.01% Tween20 and 0.2% BSA. Cells were incubated with primary antibodies for 2 h at RT, washed three times with TBS-T and incubated with anti-rabbit Alexa Flour 546 or anti-mouse Alexa Flour 488 secondary antibodies (Molecular Probes, Invitrogen, UK) for 1 h at RT in the dark. Cells were washed three times with TBS-T and mounted using ProLong Gold antifade reagent (Invitrogen, UK). Images were obtained using a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Inc., USA).

RNA isolation, RT-PCR and Real-Time PCR

A normal human tissue RNA panel was purchased from BioChain Institute Inc. (CA, USA). Total RNA was purified using

Trizol reagent (Invitrogen, UK) following the manufacturer's recommendations. The RNA concentration was determined using a NanoDrop ND-1000 instrument (Thermo Scientific, USA). cDNA was synthesized from DNase-treated (Ambion, Invitrogen, UK) RNA with Superscript III (Invitrogen, UK) and mixture of oligo dT and random hexamers, according to the manufacturer's recommendations. RT-PCR was carried out using HOT FIREpol® Master Mix (Solis Biodyne, Estonia). Real-time PCR (qRT-PCR) was performed in triplicate using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, UK) and the LightCycler® 480 Real-Time PCR System (Roche Applied Science). The fold of change was calculated relative to the control siRNAs after normalization to *GAPDH* expression. Primer sequences are listed in Table S2.

Protein extraction and immunoblot

TAF4 or control siRNA-treated cells were collected by trypsin-EDTA (PAA Laboratories) and washed once with ice-cold 1xPBS. Cell fractionation was carried out according to modified Dignam protocol [35] using 0.2% Nonidet P-40 in the lysis buffer as described by Kazantseva et al. [36]. Total protein concentration of nuclear lysates or whole cell extracts was measured using a BCA Protein Assay kit (Pierce Biotechnology Rockford, IL, USA). Equal amounts of total protein in a nuclear lysate or a whole cell extract were separated on 10% polyacrylamide gel and blotted to PVDF membrane (GE Healthcare). The membrane was treated as described [37] using a non-blocking technique. The following antibodies were used: TAF4 (BD Biosciences, 612054), CDKN1A (Santa Cruz, sc-756), $TP53^{Ser15}$ (Cell Signaling, 9284), ADIPOQ (Chemicon, MAB3604), PPARG2 (Chemicon, MAB3872), RUNX2 (Abcam, ab76956), OPN (Santa Cruz, sc-10591), COL21A (Millipore, MAB1330), MMP13 (Biomol, SA-371), SOX9 (Millipore, AB5535), ß-catenin (Santa Cruz, sc-7963) and GAPDH (Sigma, G8795) antibodies. Secondary HRP-conjugated antibodies were purchased from Abcam (UK). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Rockford, IL, USA).

WST-1 Cell Proliferation Assay

hMSCs were grown in 96-well flat bottom tissue culture plates to 80% confluency and treated with *TAF4* or control siRNA. WST-1 reagent (Roche Applied Science) was added following the manufacturer's instructions and plates were returned to 37°C for 2 h. Dye conversion was measured using SPECTRAmax 340 PC Microplate Reader (Molecular Devises LLC, USA) and the data were analyzed using Softmax Pro 3.12 software. Cell viability was evaluated every 24 h, up to 96 h post-treatment.

Cell cycle analysis

hMSCs after siRNA treatments were harvested by trypsinization, washed with 1xPBS and counted using a NucleoCounter NC-100 (Chemometec, Denmark). Approximately 4×10^5 cells were resuspended in 1 ml of 1xPBS containing 2% FBS and fixed with ice-cold ethanol (70% v/v) overnight at -20°C. Cell pellets were exposed to 100 µg/ml of RNaseA (Fermentas, Thermo Scientific) and 40 µg/ml of Propidium Iodide (AppliChem GmbH) in 1xPBS for 30 min at RT in the dark. Cell cycle distribution was assessed using Accuri C6 flow cytometer (BD Biosciences). For each sample, 10 000 individual events were collected.

Senescence-associated ß-galactosidase assay

Senescence-associated ß-galactosidase (SA- ß-gal) activity was examined as previously described [38]. Cells treated with 1 µM 4-NQO (Sigma) were used as a positive control. SA- ß-gal activity was analyzed 48 h after siRNA treatment.

Results

Tissue- specific alternative splicing of human *TAF4* targeting TAFH domain

To identify tissue-specific expression patterns of human *TAF4* mRNA splice variants, RT-PCR method with subsequent sequencing analyses of PCR fragments was used. Sequence analysis of the RT-PCR products revealed a variety of tissue-specific splice variants preserving the reading frame (**Figure 1**). However, a significant number of *TAF4* alternatively spliced mRNAs contained a premature termination codon, indicating that these splice variants are subject to nonsense-mediated RNA decay (data not shown). Tissue-restricted splicing patterns of *TAF4* containing in-frame splicing events encoding protein isoforms are shown in **Fig. 1A**. Schematic presentation of ten different TAF4 isoforms encoded by different splice variants is shown in **Figure 1B**. Interestingly, alternative splicing frequently targets exons VI and VII encoding the hTAF4-TAFH domain (**Fig. 1B**). As shown in **Figure 1**, the structure of the hTAF4-TAFH domain is apparently different in all isoforms except *TAF4_v1* and *TAF4_v3*, as evidenced by sequence analysis of identified *TAF4* mRNA alternative splice variants (ASVs) containing exonal deletions (*TAF4_v2, TAF4_v4-9*) or extensions (*TAF4_v10*) with ORF preservation. Transcripts *TAF4_v3* and *TAF4_v8* differ also in their N-terminal part.

In many tissues examined, certain alternatively spliced *TAF4* mRNAs were expressed approximately at the same levels. Two *TAF4* ASVs, *TAF4_v1* and *TAF4_v2*, were the most abundant and the most broadly expressed splice variants detected in all tissues analyzed (**Fig. 1A**). *TAF4_v1* corresponds to the longest transcript (GenBank *NM_003185.3*). Alternatively spliced *TAF4* mRNAs with altered hTAF4-TAFH domains exhibit distinct patterns of tissue-specific expression. Alternatively spliced *TAF4_v2* mRNAs containing simultaneously a deletion of exons VI – IX and an in-frame stop codon in the exon X encode TAF4 protein isoforms with the entire hTAF4-TAFH domain deleted. The ratio and levels of *TAF4_v1* and *TAF4_v2* expression varied across tissues with *TAF4_v2* dominating in bone marrow, kidney, ovary, placenta, prostate, testis and thymus and barely detectable in colon, skin and spleen. Other alternative mRNA splice variants of *TAF4* were highly tissue-specific. *TAF4_v4* mRNAs lacking exon VII that encodes a part of the hTAF4-TAFH domain were identified only in ovary, placenta, stomach, testis and thymus (**Fig. 1A**). Expression of *TAF4_v5* mRNAs with deleted exon VI encoding the major part of the hTAF4-TAFH domain overlapped with expression of *TAF4_v4* in all tissues except for ovary (**Fig. 1A**).

Differentiation of mesenchymal stem cells to adipocytes, osteoblasts and chondrocytes is associated with expression of *TAF4* transcripts with the deleted hTAF4-TAFH domain

Analysis of expression of *TAF4* splice variants revealed dominant expression of *TAF4_v1* in proliferating hMSCs (**Fig. 2A**). Splice variant *TAF4_v2*, which encodes a protein isoform with the entire hTAF4-TAFH removed, showed different expression in hMSCs isolated from different individuals. It was

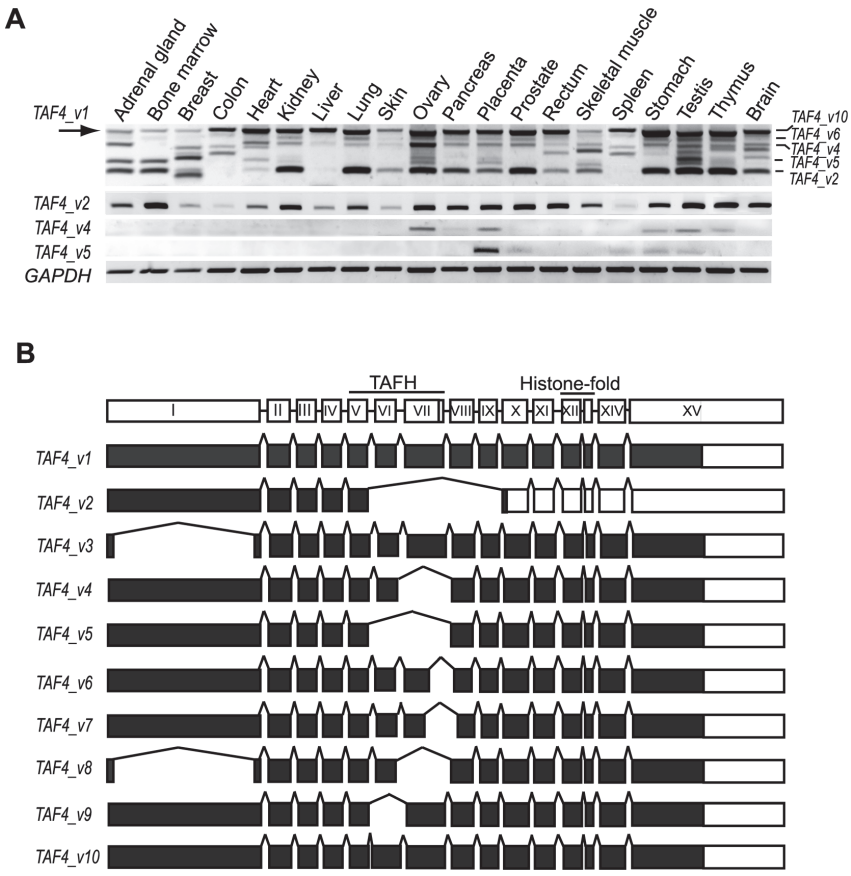

Figure 1. Expression analysis of *TAF4* alternative splice variants in human tissues. (**A**) RT-PCR analysis of human tissues using *TAF4* transcript-specific primers. Primers for the full-length *TAF4_v1* amplify all splice variants, whereas ASV-specific primers generate predominantly one PCR product. The numbers of PCR cycles and exposure times of the images for each set of primers vary and cannot be directly compared. The arrow indicates the canonical *TAF4_v1* ASV. (**B**) Schematic representation of the human *TAF4* gene structure and its alternative splice variants drawn in scale. The regions encoding the respective domains are indicated above the gene structure. Filled boxes represent the coding regions of the ASVs.
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detected at a low level only in a few hMSC isolates, indicating that *TAF4_v2* is a rare transcript (**Fig. 2A**). Alternative splice variants of *TAF4* encoding for proteins with a structurally altered hTAF4-TAFH domain become more abundant in the course of differentiation of hMSCs into adipocytes, osteoblasts and chondrocytes (**Fig. 2B**). Expression of *TAF4_v2* ASV was observed in all differentiated hMSCs together with *TAF4_v1* and other splice variants (**Fig. 2B**). The data clearly shows that hMSC differentiation along adipo-, osteo- and chondrogenic lineages was accompanied by changes in the expression of *TAF4* mRNA splice variants with a structurally altered hTAF4-TAFH domain.

RNAi analysis was carried out using two structurally different siRNAs targeting exons V or VI in N-terminal half of the hTAF4-TAFH domain of *TAF4* to evaluate the role of the hTAF4-TAFH domain in differentiation of hMSCs (**Fig. 2C**). We verified, using semi-quantitative RT-PCR and Western blot analysis, that the siRNAs silenced their corresponding exon-containing transcripts efficiently (**Fig. 2C, D**). Both, *TAF4_ex5_siRNA* and *TAF4_ex6_siRNA* resulted in a significant down-regulation of their transcriptional target, *TAF4_v1*, at the mRNA and protein levels as compared to cells transfected with control siRNAs only (**Fig. 2C, D** and data not shown). As both siRNAs had similar effects on transcriptional silencing of *TAF4_v1*, we further refer to *TAF4_ex5_siRNA* or *TAF4_ex6_siRNA* as *TAF4* siRNA. Intriguingly, silencing of *TAF4_v1* resulted in the up-regulation of alternatively spliced *TAF4_v2* and *TAF4_v5* mRNAs and decreased expression of *TAF4_v4* mRNA levels (**Fig. 2C**) suggesting a feedback loop in controlling alternative splicing of *TAF4*. Prolonged treatments of hMSCs with *TAF4* siRNAs resulted in the induced expression of *TAF4_v5* mRNAs and significantly reduced expression of *TAF4_v4* mRNAs. However, this alternative splice profile is individual-specific and each donor has its own TAF4 ASVs composition. Western blot analysis data revealed that hMSCs expressed the canonical form of TAF4 protein at relatively low levels, whereas

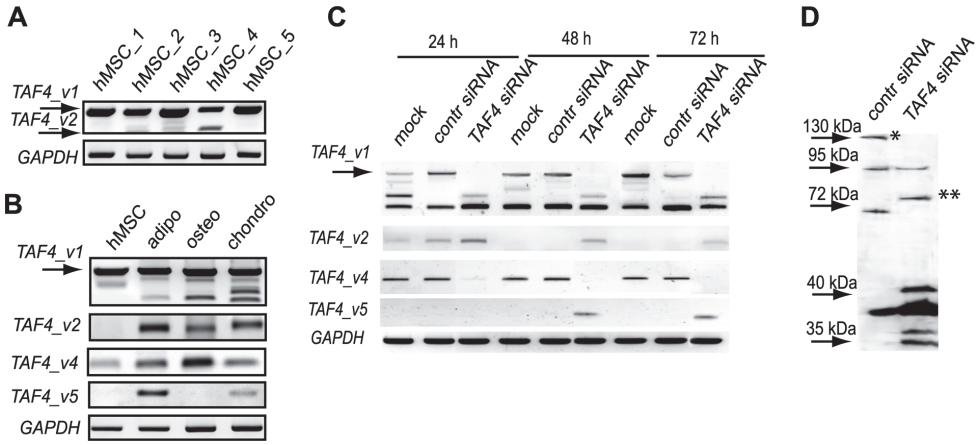

Figure 2. Expression of TAF4 splice variants and isoforms in human MSCs differentiated into adipocytes, osteoblasts or chondrocytes and treated with *TAF4* RNAi. (**A**) RT-PCR analysis of different individual hMSCs clones (hMSCs 1–5) using *TAF4_v1a* (full length) specific primers (see Table S2). Expression of *GAPDH* mRNA is shown at the bottom. (**B**) Expression of *TAF4* ASVs encoding proteins with compromised hTAF4-TAFH domain is dominant in differentiated hMSCs. RT-PCR analysis using *TAF4* ASV-specific primers was performed 7 days after induction of differentiation of hMSCs towards adipo-, osteo- and chondrogenic lineages. *GAPDH* mRNA expression was used for the normalization. (**C**) Expression of *TAF4* ASVs following treatment of human MSCs with control and hTAF4-TAFH-domain targeting *TAF4* siRNAs. Cells were treated with *TAF4* or control siRNAs at the indicated time points and RT-PCR analysis performed using *TAF4* ASV-specific primers. Analysis of *GAPDH* mRNA expression was used for normalization. (**D**) siRNA-mediated silencing targeting *TAF4* exons V or VI induces changes in the expression of TAF4 protein isoforms as detected at 48 h post-treatment using Western blot analysis. The asterisk indicates the canonical form of TAF4 protein with the molecular weight of 135 kDa, two asterisks indicate TAF4_v2 isoform with a calculated molecular weight of about 73 kDa.
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upon RNAi targeting of the hTAF4-TAFH domain the pattern of TAF4 isoforms changed significantly (**Fig. 2D**). As evidenced in **Figure 2D,** the RNAi induced changes could involve the most abundant isoform TAF4_v2 with the calculated molecular weight of 73 kDa. Given that TAF4 isoforms (TAF4_v1, _v4, _v5, _v6, _v7, _v9) have relatively similar calculated molecular weights, the Western blot resolution was insufficient to make conclusive identification of these isoforms upon RNAi. Accordingly, siRNAs targeting exons V and VI shifted *TAF4* mRNA splicing patterns in human MSCs towards the generation of mRNAs encoding protein isoforms with an altered hTAF4-TAFH domain.

hTAF4-TAFH controls proliferation and cell cycle exit via TP53 activation and switch from canonical to non-canonical WNT signaling

Next, we analyzed the effect of the hTAF4-TAFH domain-targeting RNAi on the proliferation and cell cycle of hMSCs (**Fig. 3**). Silencing of alternative splice variants encoding a structurally intact hTAF4-TAFH domain in hMSCs resulted in growth inhibition as analyzed using WST-1 proliferation assay (**Fig. 3A**). Already 6 h treatment of hMSCs with *TAF4* siRNAs resulted in the upregulation of *CDKN1A* and *CDK2* levels (**Fig. 3B**). Western blot analysis revealed that changes in *TAF4_v1* and *CDKN1A* mRNA levels were accompanied by a marked decrease in the expression of the canonical form of TAF4 protein and significant increase of CDKN1A protein levels. Also, we detected accumulation of hyperphoshporylated $TP53^{Ser15}$ in hMSCs following 24 h treatment (**Fig. 3C).** At 48 h post-treatment of hMSCs with *TAF4* siRNAs, increased *TP53* transcription was observed (**Fig. 3C**). These findings demonstrate that depletion of the canonical hTAF4-TAFH activity in hMSC correlates with the cell cycle exit.

Further, we examined whether the observed cell-growth retardation was related to induction of apoptosis. Flow cytometry analysis data clearly showed that the proportion of apoptotic cells in *TAF4* siRNA treated hMSCs was insignificant (**Fig. 3D**). Propidium iodide staining analysis using the NucleoCounter (data not shown) confirmed these data. To exclude that *TAF4* siRNA triggers TP53-dependent senescence in the human MSCs, quantitative senescence-associated ß-galactosidase assay was performed. Assay results clearly show no increased ß-galactosidase activity in *TAF4* siRNA treated hMSCs compared to control siRNAs at 48 h post-treatments (**Fig. 3E**). Also, no changes in hMSC morphology were observed following *TAF4* siRNA treatments (data not shown). These results suggest that observed suppression of cell proliferation is associated with cell cycle arrest and not with the induction of cell senescence or apoptosis. Obtained results support the hypothesis of TP53 involvement in TAF4-driven differentiation of hMSC.

Since TP53 activates WNT pathway signaling in mouse embryonic stem cells [39] and the hTAF4-TAFH domain is the direct target of WNT signaling in *Drosophila* [19], we examined the possibility that the WNT pathway is also involved in hTAF4-TAFH governed hMSC proliferation and differentiation. We found that *TAF4* siRNA treatment significantly down-regulated the major player in canonical WNT signaling, ß-catenin, while the expression levels of non-canonical WNT signaling activator *WNT5A* and the inhibitor of the WNT pathway *DKK1* were significantly increased (**Fig. 3F**). Taken together, these findings provide the first cues that TAF4 protein isoforms with a deleted hTAF4-TAFH domain may function as direct co-activators in the non-canonical WNT signaling pathway that is mediated by JNK, PKC, Ca (2+) or Rho [40].

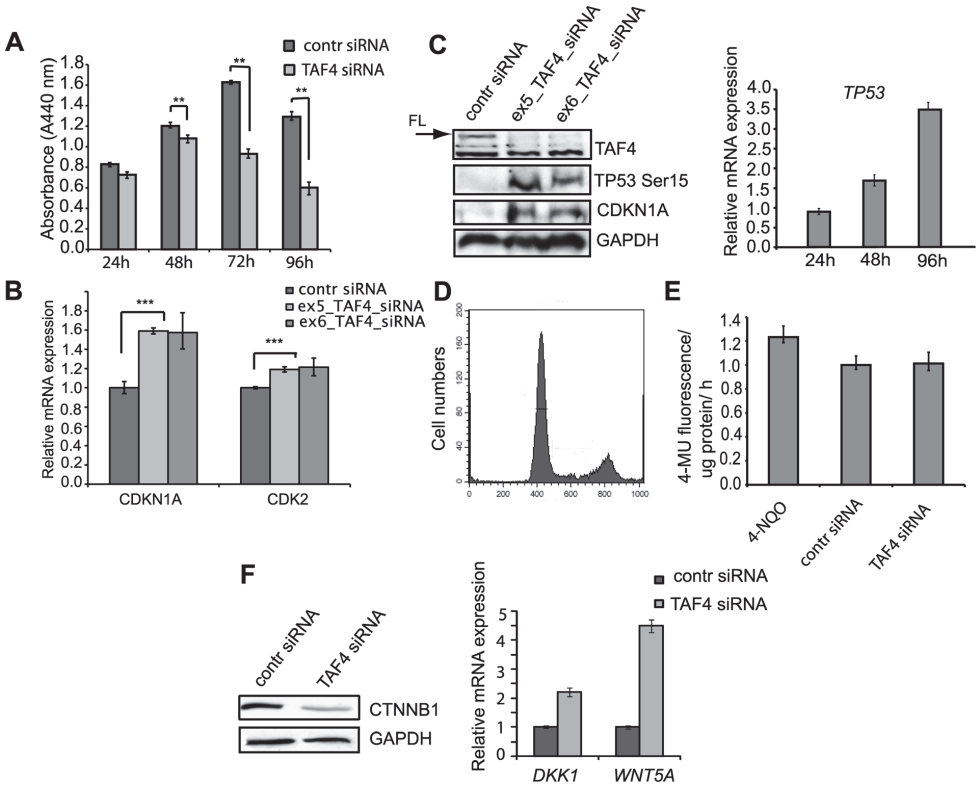

Figure 3. ***TAF4*** **siRNA treatment of human MSCs induces TP53-dependent cell-cycle arrest and switching from canonical to non-canonical WNT signaling.** (**A**) Effects of *TAF4* siRNA treatments on hMSC proliferation at different time points as compared to negative control siRNA. hMSCs were transfected with 50 nM of *TAF4* or control siRNAs and analyzed at the indicated time points by WST-1 proliferation assay. Experiments were done in triplicate and a comparison was made to control siRNA treatments (** indicates significant differences between control and *TAF4* siRNA groups with $P<0.01$). (**B**) Down-regulation of the canonical form of TAF4 affects the expression of cell cycle regulators. hMSCs were transfected with 50 nM *TAF4*-specific (ex5_ or ex6_*TAF4* siRNAs) or control siRNAs. Relative expression of cell cycle regulators *CDKN1A* and *CDK2* as compared to control siRNAs transfected cells was analyzed using real-time RT-PCR at 6 h post-treatment. Observed differences were statistically significant (Student's *t*-test) with *** $P<0.001$. (**C**) *TAF4* siRNA-mediated RNAi affects expression of cell cycle regulator proteins. Western blot analysis of cell cycle regulators TP53 and CDKN1A/P21 24 h following transfection of hMSCs with control or both, ex5_ and ex6_*TAF4*-specific siRNAs. Expression of TAF4 and GAPDH was analyzed to ensure effective silencing and equal loading *(left)*. Time-dependent expression of *TP53* was analyzed by real-time PCR and compared to the mRNA levels in *TAF4* and control siRNA treated hMSCs *(right)*. (**D**) Down-regulation of the canonical form of TAF4 does not induce apoptosis. FACS analysis of the cell cycle progression of hMSCs treated with *TAF4* siRNAs. (**E**) *TAF4* siRNA treatment doesn't cause senescence of hMSCs. Quantitative SA-ß-gal assay. hMSC extracts were prepared from *TAF4* and control siRNA transfected cells 48 h post-treatment. 1 μM 4-NQO was added to hMSCs for 1 h and used as positive senescence control. Fluorescence intensity of 4-MU hydrolysis was normalized to total protein. Error bars in experiments represent the standard deviations of three independent experiments ($P<0.005$) (**F**) RNAi of hTAF4-TAFH switches from canonical to non-canonical WNT signaling. 20 μg of control or *TAF4* siRNA-treated whole cell lysates were analyzed by Western blot analysis for the expression of ß-catenin (CTNNB1) and GAPDH as loading control *(left)*. Real-time PCR shows increased expression of non-canonical markers of WNT signaling in *TAF4* siRNA treated hMSCs as compared to control siRNA treated cells. Differences are statistically significant with $P<0.001$ *(right)*.

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hTAF4-TAFH domain integrity supports adipo- and osteogenic and blocks chondrogenic differentiation of hMSCs

hMSCs were differentiated along adipo-, osteo- and chondrogenic lineages upon treatments with *TAF4* siRNAs or control siRNAs following analysis of expression of appropriate lineage-specific markers using Western blot and quantitative RT-PCR to analyze the effects of hTAF4-TAFH on the differentiation potential of hMSCs. Effective downregulation of the canonical form of TAF4 protein along with the hyperphoshporylation of $TP53^{Scr15}$ was observed in siRNA treated cells (**Fig 4 A–C**). In addition, RNAi treatments followed by differentiation resulted in the induction of expression of *TAF4* splice variants encoding protein isoforms with a modified hTAF4-TAFH domain (**Fig 4 A–C**).

On day 6 of adipogenic differentiation, specific Oil-Red-O staining revealed intensely reactive lipid droplets in control vehicle-treated cells (**Fig. 4A**), indicating adipogenic differentia-

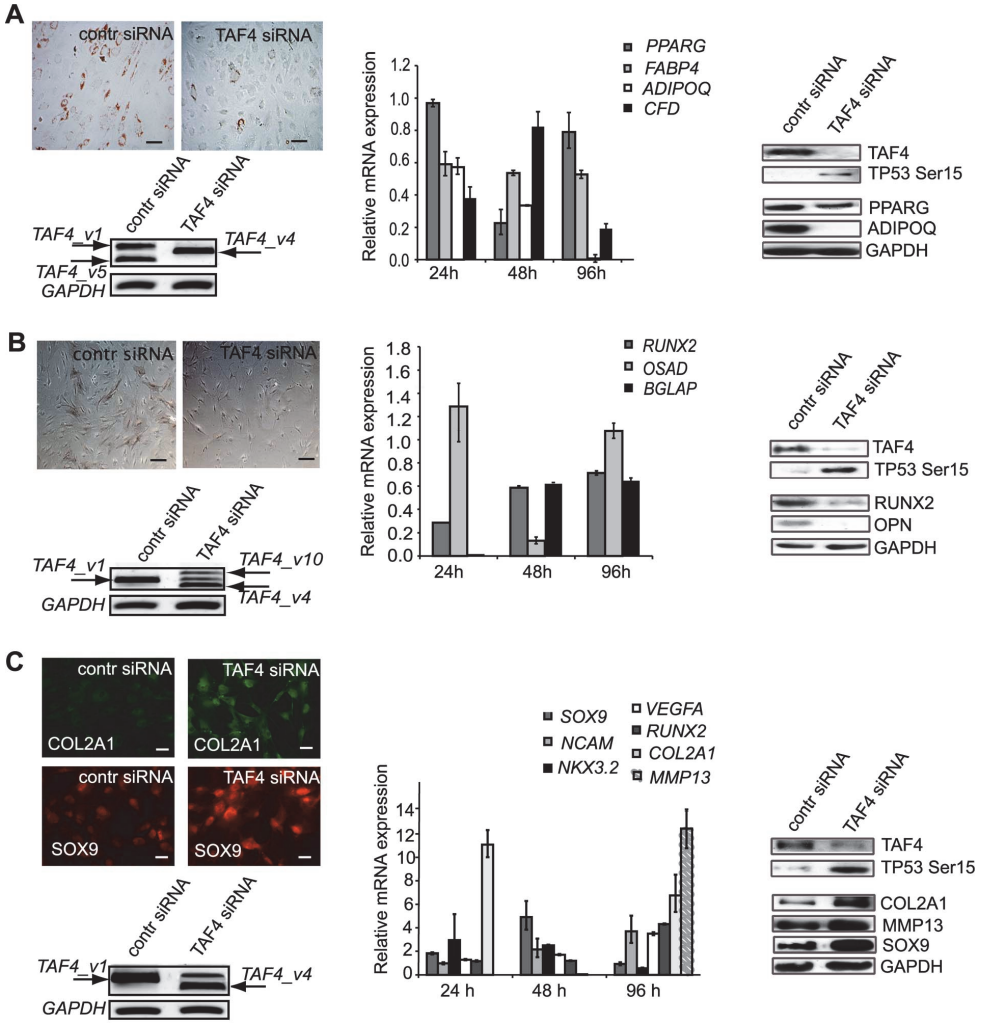

Figure 4. TAFH domain disruption by *TAF4* siRNA attenuates adipogenesis and osteogenesis and accelerates chondrogenesis in human MSCs. (**A**) TAF4 down-regulation slows down adipogenesis in hMSCs. Oil-Red-O staining of lipid droplets of *TAF4* or control siRNA treated and adipogenesis stimulated hMSCs at day 6 post-induction. siRNAs were transfected 24 h prior to the stimulation of differentiation of hMSCs. Scale bar, 40 μm *(top left)*. Western blot analysis reveals reduced expression of adipogenic markers, namely PPARG and ADIPOQ at day 5 after induction of differentiation of *TAF4* siRNA treated hMSCs along adipocyte pathway *(right panel)*. Real-time PCR analysis of the expression of adipocyte markers in hMSCs at different time points of post-transfection and differentiation. The expression is normalized to control siRNA treatments *(middle panel)*. RT-PCR analysis of *TAF4* ASVs expression using *TAF4_v1b* specific primers in *TAF4* or control siRNA transfected and towards adipogenesis stimulated hMSCs at day 5 post-transfection. *GAPDH* mRNA was analyzed for gel loading normalization *(left bottom)*. (**B**) RNAi of hTAF4-TAFH inhibits osteogenic differentiation of hMSCs. Alkaline phosphatase staining of *TAF4* or control siRNA exposed and osteogenesis stimulated hMSCs at day 6 post-induction. siRNAs were transfected 24 h prior to the stimulation of hMSC differentiation. Scale bar, 40 μm *(top left)*. Western blot analysis reveals reduced expression of osteogenic markers RUNX2 and OPN *(right panel)*. Real-time PCR analysis of the expression of osteogenic markers in hMSCs at different time points post-transfection and differentiation. The expression is normalized to negative control siRNA treatment *(middle panel)*. RT-PCR analysis of *TAF4* ASVs expression using *TAF4_v1b* specific primers in *TAF4* or control siRNA treated and stimulated to osteogenic differentiation hMSCs at day 5 post-transfection. *GAPDH* mRNA was analyzed for gel loading normalization *(left bottom)*. (**C**) RNAi of hTAF4-TAFH accelerates chondrogenic differentiation of hMSCs. Immunofluorescence staining analysis of *TAF4* or control siRNA treated hMSCs upon chondrogenic stimulation at day 6 post-treatment reveals induced expression of COL2A1 and SOX9. siRNAs were transfected 24 h prior to stimulation of differentiation of hMSCs. Scale bar, 40 μm *(top left)*. Western blot analysis shows increased expression of chondrogenic marker genes in *TAF4* siRNA treated hMSCs. COL2A1 expression was upregulated at day 2 post-differentiation; SOX9 and MMP13 level of expression were increased at 8 days after siRNA treatments *(right panel)*. Real-time PCR analysis of the expression of chondrogenic markers in hMSCs at different time points post-transfection and differentiation. The

expression is normalized to control siRNA treatment *(middle panel)*. RT-PCR analysis of *TAF4* ASV expression using *TAF4_v1b* specific primers in *TAF4* or control siRNA treated hMSCs upon stimulation to chondrogenic differentiation at day 5 post-transfection. *GAPDH* mRNA was analyzed for gel loading normalization *(left bottom)*. Effects of RNAi of hTAF4-TAFH on activation of $TP53^{Ser15}$ were observed in each differentiation study using immunoblot analysis. Real-time PCR differences were found to be statistically significant with $P<0.005$.
doi:10.1371/journal.pone.0074799.g004

tion, whereas far less intensive staining was observed in *TAF4* siRNA transfected hMSCs. Analysis of expression of adipose-specific PPARG and ADIPOQ at protein and RNA levels indicated a significantly delayed adipogenic differentiation of hMSCs exposed to *TAF4* siRNAs targeting hTAF4-TAFH (**Fig. 4A**). These findings show that RNAi silencing of hTAF4-TAFH activity blocks adipogenesis in hMSCs.

To assess the effect of hTAF4 RNAi on osteogenic differentiation, hMSCs were treated with *TAF4* or control siRNAs and cultured in osteogenic differentiation medium for 5 days. Alkaline phosphatase (AP) staining confirmed effective osteogenic differentiation in control siRNA-treated cells, whereas much less AP + cells were observed in *TAF4* siRNA treated hMSCs upon differentiation (**Fig. 4B**). Also, immunoblot analysis using anti-RUNX2 and anti-OPN antibodies showed decreased levels of expression of these osteogenic markers in *TAF4* siRNA transfected hMSCs upon differentiation (**Fig. 4B**). Surprisingly, the expression of *RUNX2* and *BGLAP* mRNAs that initially (24 h post-treatment) decreased significantly, reached the control level by day 5 of differentiation. Substantially decreased levels of *OSAD* expression were detectable only 48 h post-differentiation (**Fig. 4B**). All the data evidenced severely delayed osteogenesis in hMSC cells with low hTAF4-TAFH activity.

Next, we examined the role of hTAF4-TAFH in chondrogenic differentiation. Immunofluorescence analysis of *TAF4* and control siRNA-treated hMSCs that were subjected to chondrogenic differentiation for 6 days showed intense staining with anti-COL2A1 and anti-SOX9 antibodies in TAF4-depleted hMSCs, indicating enhanced chondrogenic differentiation in these cells (**Fig. 4C**). In close agreement with these results, immunoblot analysis data further confirmed the up-regulation of COL2A1 protein expression by day 1, SOX9 expression by day 2 and MMP13 expression by day 5 (**Fig. 4C**), supporting the evidence of accelerated chondrogenesis. To analyze the expression of chondrogenic markers upon *TAF4* siRNA treatments, a real-time RT-PCR analysis was performed using a set of gene-specific primers. Increased levels of *SOX9* mRNA, with a peak at day 2, rapid upregulation of *NKX3.2* and *COL2A1* mRNAs by day 1, gradually rising levels of expression of *NCAM, VEFGA, RUNX2* mRNAs and the appearance of *MMP13* mRNA later in the differentiation process were observed in *TAF4* siRNA treated cells compared with that of the control vehicle treated hMSCs upon chondrogenic differentiation (**Fig. 4C**). Thus, based on the immunofluorescence and gene expression analysis data it was concluded that the activity of the canonical hTAF4-TAFH suppresses chondrogenesis.

Notably, the levels of *TAF4_v1* mRNA decreased substantially whereas those of *TAF4* mRNAs encoding protein isoforms with a modified hTAF4-TAFH domain (*TAF4_v2*, *TAF4_v4*, *TAF4_v10*, and other rare transcripts (data not shown)) accumulated upon *TAF4* RNAi treatments and during the further course of differentiation of hMSCs (**Fig. 4A–C**). The data is in close agreement with data on the expression of *TAF4* splice variants in the differentiated hMSCs (**Fig. 2B**), which altogether strongly support the herein described findings that hTAF4-TAFH domain integrity is required for adipo- and osteogenic differentiation and controls chondrogenic differentiation.

Discussion

Here, we present the first description of alternatively spliced *TAF4* mRNAs carrying deletions of exons encoding the hTAF4-TAFH domain. We describe the functional consequences of the structural integrity of the hTAF4-TAFH domain on cellular differentiation. Our data reveals that differentiation of hMSCs along adipo-, osteo- and chondrogenic lineages is, at least in part, regulated by hTAF4-TAFH domain activity, with possible cross-talks to the early activity of TP53 and switching of WNT signaling from a canonical to a non-canonical pathway.

Variety of hTAF4 AS variants

Our data, along with published findings [30], shows a broad distribution of alternative splice variants across cells and tissues of *TAF4* alternative mRNAs. Here, we show that across the *TAF4* gene, alternative splicing predominantly targets exons VI and VII. These exons encode the hTAF4-TAFH domain, a five-helix structure that is responsible for protein-protein interactions and recognizes a hydrophobic DΨΨζζΨΦ motif (similar to LxxLL) of TAF4 interaction partners [18]. Alternative splicing of exons VI and VII alters the flat and wide binding surface in the hTAF4-TAFH domain, making it more similar to that of the ETO-TAFH domain. ETO-TAFH-dependent interactions with LxxLL-carrying proteins, including LZIP, E-proteins, nuclear hormone receptors and subunits of Mediator complex have been suggested to affect the whole PIC composition and activity [41]. Thus, splicing events in the exons coding the hTAF4-TAFH domain ultimately contribute to the changes in target specificity, perhaps allowing the fine-tuning of a transcriptional response to activators that are important during development. Close support for this assumption stems from our data showing that in response to *TAF4* RNAi treatments, hMSCs start to express alternative splice variants of *TAF4*, namely *TAF4_v2*, *TAF4_v4* or *TAF4_v5* mRNAs that encode protein isoforms with only 2 helixes out of five preserved from the canonical hTAF4-TAFH structure. It is highly likely that changed co-activator properties of the hTAF4-TAFH domain may influence overall TFIID complex stability, PIC assembly and the basal transcription, having functional consequences in the selection of developmental pathways in human cells.

It requires further clarification, whether *TAF4* alternative splice variants with ORF preservation will encode functional proteins or could act as lncRNAs. In both cases, *TAF4* ASVs impact the ability of TAF4 to promote the cell cycle and control differentiation. ASVs retaining canonical ORF are co-expressed although at different levels than *TAF4* ASVs encoding protein isoforms with altered hTAF4-TAFH activity. For example, the expression levels of *TAF4_v1* mRNA encoding the canonical form of the protein with intact hTAF4-TAFH are comparable with the sum of other ASVs in differentiated hMSCs. Therefore, it is likely that the relative levels of *TAF4* ASVs and mutual cross-talk (cooperation or competition) will influence the final biological outcome. The expression of some *TAF4* ASVs is ubiquitous while of the others are tissue-specific (our data). Differences in the levels of expression of *TAF4* ASVs were observed in all individuals analyzed (**Fig. 2A**) and distinguished by every hMSC donor [42]. In addition, differences in silencing or activation of various *TAF4* ASVs were also observed throughout the study (**Fig. 2A, 4**). These individual-

specific diversity could be related to the differences in the chromatin and epigenetic background as it has been shown that siRNAs that target exonic sequences in the close proximity to alternatively spliced exons could regulate splicing in a chromatin and epigenetic context-dependent manner [43].

Therefore apparently, the numerous *TAF4* ASVs with altered hTAF4-TAFH activity may permit a differential regulation of TAF4 functions during cell differentiation. Yet, this hypothesis awaits further clarification.

Molecular mechanisms of hTAF4-TAFH-activity-mediated differentiation

hMSCs that were differentiated into adipo-, osteo- or chondrocytes showed complex expression patterns of *TAF4* ASVs encoding proteins with altered hTAF4-TAFH. Recently, using modified versions of TAF4 protein it was shown that ETO-TAFH domain of TAF4 is targeted by WNT signaling in *Drosophila* [44], [19]. In addition to global gene silencing that has been evidenced to occur during ES cell differentiation [45], proteasome-dependent TAF4 degradation was observed in F9 embryonic carcinoma cells in response to retinoic acid-induced differentiation [46]. As these processes are related to cell fate determination and control of stem cell proliferation, this prompted us to investigate the consequences of hTAF4-TAFH inactivation on hMSCs proliferation and differentiation.

RNAi targeting of exons V and VI of *TAF4* resulted in cell cycle arrest and accumulation of hyperphosphorylated $TP53^{Ser15}$ protein in hMSCs. TP53 activation via phosphorylation is associated with the induction of apoptotic cell death or irreversible cell-cycle exit, commonly termed cellular senescence [47]. Both of these processes are linked to cellular differentiation. Depending on the state and cellular environment, TP53 exerts a regulatory role on various differentiation programs [48–54]. Induction of TP53 expression represses cell proliferation and through *miR-34a* and *miR-145* activation downregulates pluripotent stem cell factors, such as OCT4, KLF4, LIN28A, and SOX2 thereby affecting differentiation and human cell state [55]. Our data on the interrelations of TP53 and TAF4 are in close agreement with published data related to TFIID complex subunits. The detailed mechanism of TAF4-TP53 interaction remains to be established, but our data allows for suggesting the involvement of TP53 in TAF4-dependent differentiation of hMSCs. Indeed, TAF4 was detected on a TP53-binding site on a *CDKN1A/P21* promoter and its binding to the promoter increased in response to UV irradiation [56]. TAF9 has been shown to regulate the stability and activity of TP53 by binding to its N-terminally located transcription activation domain [57,58]. Functional interactions between TP53 and TAF3 [59], TAF1 [60], TAF6 [61] and TBP [62] have been reported. Altogether, the data suggests that while TAF4 may be involved in the stimulation of cell differentiation by alternative splicing generating protein isoforms with varying hTAF4-TAFH, the down-stream activation of p53 pathways executing this differentiation process are likely to play an important role.

WNT signaling promotes activation of WNT target genes by targeting Pygopus-TAFH interactions in *Drosophila* [44]. Active canonical WNT signaling stimulates osteogenesis in certain cellular contexts [63,64]. However, interplay between canonical ß-catenin-dependent and a variety of non-canonical pathways has been evidenced to guide cells to differentiate along defined pathways and also directs cell fate decisions of hMSCs [40]. Several members of the WNT family have been shown to block osteogenesis and adipogenesis. For example, a non-canonical ligand WNT5A inhibits the ability of PPARG to activate its target genes and ultimately thus prevents adipogenesis [65]. The positive impact of WNT inhibitor DKK1 on early chondrogenesis has also previously been documented [66]. Furthermore, to confirm the interplay of TP53 and WNT signaling that was observed by us in *TAF4* siRNA treated cells, previous studies have shown that TP53 and its target *miR-34* suppress canonical WNT signaling [67]. Our data showing reduced levels of ß-catenin in response to decreased expression of *TAF4_v1* with hTAF4-TAFH intact are in close agreement with the findings described above. Taken together, we suggest that abrogation of hTAF4-TAFH activity by expression of TAF4 protein isoforms with an hTAF4-TAFH altered structure has severe consequences on the co-activator function of TAF4 in canonical WNT signaling. In order to counter-balance the inhibition of the canonical WNT pathway, compensation by activation of the non-canonical pathway by WNT5A takes place and acts as a mediator of the induction of chondrogenesis in hMSCs upon differentiation. Similar findings have been described by Bradley et al [68].

RNAi targeting of hTAF4-TAFH activity results in down-regulation of *TAF4_v1* and promotes chondrogenesis by inhibiting or delaying osteo- and adipogenesis of human adipose derived hMSCs. Differentiation along osteo- and adipogenic lineages verified by using expression analysis of appropriate markers was delayed but not completely inhibited upon RNAi targeting of hTAF4-TAFH activity. Since close interplay between osteogenesis and chondrogenesis is regulating the early development of bone, and RUNX2 modulates both of these differentiation programs, it is also possible that downregulation of hTAF4-TAFH activity in hMSCs influences *RUNX2* turnover in osteogenesis. Also, TP53 deficiency has been shown to enhance osteogenesis via SMAD1 signaling in mice [69], thereby providing a strong possibility that low levels of hTAF4-TAFH activity promote a chondrogenic switch in mesenchymal cells with activated TP53.

Depletion of hTAF4-TAFH is necessary for normal development

Inactivation of individual TAFs in *Drosophila* and mammalian cells have demonstrated that TAFs are not essential for the transcription of all RNA pol II-dependent genes and in fact, there is a great variation in target genes of individual TAFs [28], [70–72]. For example, myoblasts shed most of the subunits of TFIID complex, apart from TAF3 and a TBP homolog TRF3, in the process of differentiation to myotubes [73,74]. Expression of several TAF subunits, with the exception of TAF8, was observed to be downregulated upon differentiation of 3T3-L1 preadipocytes into adipocytes [75]. Inactivation of TAF10 affects liver development and stability of the TFIID complex as a whole [76]. Furthermore, TAF7 has recently been shown to be necessary for mouse embryonic development but not for the survival and differentiation of mature T cells [21]. In *Drosophila*, targeting of TAF4 activity by RNAi yields in the reduced levels of TBP, TAF6, and TAF9 along with a severe loss in TAF1 and TAF5 protein expression without affecting their mRNA levels [77]. Reduction of TAF4 activity has been shown to have the most dramatic effects on transcription as compared with other subunits of TFIID in *C.elegans* [23]. All the data, along with our findings on TAF4 function, are consistent with the suggestion that regulated degradation of defined TFIID subunits or controlling their activity via alternative splicing, as in case of TAF4 demonstrated here, is required for directing the normal cellular differentiation process.

In conclusion, altered splicing and regulated expression of *TAF4* alternative mRNAs encoding protein isoforms with altered hTAF4-TAFH activity govern the cell-cycle progression in hMSCs through expression of cell cycle inhibitors and TP53 activation that support realization of specific differentiation programs. Our data also reveals the potential role of TAF4 isoforms in delaying

adipogenic differentiation of hMSCs and thus contributes to the understanding of the mechanisms of obesity. In adipose-derived hTAF4-TAFH-depleted hMSCs, chondrogenesis is the most preferable differentiation program. Cellular mechanisms leading to such transitions are currently not clear and should be clarified with future studies, but our research suggests the role of TP53 along with the switching of WNT signaling from a canonical to a non-canonical pathway in response to predominant expression of TAF4 ASVs with abrogated hTAF4-TAFH activity in human mesenchymal stem cells.

Supporting Information

Table S1 Description of hMSCs clones used in the study.
(DOCX)

Table S2 List of primers used in the study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JK TN KP. Performed the experiments: JK ANK AK KT. Analyzed the data: JK KP. Wrote the paper: JK KP.

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PUBLICATION II

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TAF4 Controls Differentiation of Human Neural Progenitor Cells Through hTAF4-TAFH Activity

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Abstract Expression of general transcription factor and co-activator TAF4 varies during development and in the processes of cell differentiation with suggested connection to neurodegenerative diseases. Here, we show that expression of *TAF4* alternative splice variants is different in various regions of the human brain, substantiating the role of alternative splicing of TAF4 in the regulation of neural development and brain function. Most of the described splicing events affect the TAFH homology domain of TAF4 (hTAF4-TAFH). Besides, differentiated towards neural lineages, normal human neural progenitors (NHNPs) lose canonical full-length TAF4 isoform. To study the effects of hTAF4-TAFH splicing on neuronal differentiation, we used RNAi approach to target hTAF4-TAFH-encoding domain in NHNPs. Results show that inactivation of hTAF4-TAFH domain accelerates differentiation of human neural progenitor cells. Conversely, enhanced expression of TAF4 suppresses differentiation and keeps neural progenitor cells in a stem cell-like state. Finally, we provide data on the involvement of TP53 and noncanonical WNT signaling pathways in mediating effects of TAF4 on neuronal differentiation. Overall, our data suggest that specific isoforms of TAF4 may selectively and efficiently control neurogenesis.

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Introduction

The diversity of general transcription factor (GTF) protein isoforms generated by alternative splicing ultimately relates to their biological role and reflects the modulation of activity in a spatiotemporal manner (López 1995; Ozer et al. 2000; Upadhyaya et al. 2002; Wang et al. 2006). The complexity and dynamics of transcriptome in the human brain is highly diverse in various brain regions, across development and in pathologies (Dehay and Kennedy 2009). Global changes in alternative splicing have been identified during aging and in case of neuropathologies causing Alzheimer's disease or frontotemporal lobar degeneration (Tollervey et al. 2011). In addition, differentiation of human embryonic stem cells towards neural lineages involves different signaling activities of multiple transcriptional regulators (Erceg et al. 2009) suggesting that diversity of components of transcription regulatory complexes makes the process even more complicated.

The function of TAF4, a basic subunit of TFIID complex and a co-activator of many TFs, is regulated by alternative splicing. TAF4 isoforms exert dominant negative effects on TAF4 activity in nuclear receptor-mediated transcriptional activation (Brunkhorst et al. 2004) and together with RanBPM control neuritogenesis (Brunkhorst et al. 2005). Our recent data show that alternative splicing of hTAF4-TAFH affects lineage differentiation of human mesenchymal stem cells (hMSCs), given that TAF4 isoforms with modified hTAF4-TAFH domain induce p53-dependent cell cycle arrest and support chondrogenic differentiation of hMSCs through activation of noncanonical WNT signaling (Kazantseva et al. 2013).

Here, we studied the role of hTAF4-TAFH activity during neural differentiation applying RNAi silencing and overexpression techniques. Using normal human neural progenitor cells (NHNPs) as a model, we demonstrate that modification of hTAF4-TAFH activity is critical for neuronal and glial differentiation.

Materials and Methods

Cell Culture

NHNP cells (Lonza Inc., Walkersville, MD, USA) were cultured in suspension as neurospheres in DMEM/F-12 medium supplemented with 2 % B27, 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml LIF, and 5 μg/ml heparin, 1 % of penicillin/streptomycin in T-75 flasks in a humidified atmosphere at 37 °C and 5 % CO_2. Every 7 days, the growth medium was switched to a growth medium enriched with 1 % fetal bovine serum (FBS). After nucleofection, cells were plated to poly-D-lysine coated six-well plates in the growth medium containing 1 % FBS. The next day, the growth medium was replaced with the differentiation medium. For neuronal differentiation, the medium consisted of DMEM/F-12 enriched with 20 ng/ml ß-NGF or BDNF and 2 % B27 supplement. For glial differentiation, DMEM/F-12 supplemented with 10 % FBS was used.

siRNA Transfection and Overexpression of TAF4

siRNAs targeting hTAF4-TAFH domain were designed and used as previously described (Kazantseva et al. 2013). All experiments were done using two different siRNAs targeting exons V and VI, whereas data of RNAi targeting exon VI is provided for most data illustration. Cells were dissociated into single cell suspension, and 6×10^5 cells were transfected with 50 nM of control or *TAF4* siRNAs, or 1.0 μg of TAF4 ORF cDNA in pcDREAM2.1 (GenScript, Piscataway, NJ, USA), using the Primary Mammalian Neurons Kit (Lonza Cologne AG, Germany), according to the manufacturer's instructions.

RNA Isolation, RT-PCR, and Quantitative PCR

The human brain tissue RNA panel was a kind gift from T. Timmusk's laboratory (Tallinn University of Technology, Estonia). Total RNA from NHNPs was purified using Trizol reagent, following the manufacturer's recommendations. cDNAs were synthesized from DNase-treated RNA using Superscript III (Invitrogen, UK) and a mixture of oligo dT and random hexamers, according to the manufacturer's recommendations. RT-PCR was carried out using HOT FIREpol® Master Mix (Solis BioDyne, Estonia). Real-time PCR was performed in triplicate using Platinum® SYBR® Green qPCR SuperMix-UDG and the LightCycler® 480 Real-Time PCR System (Roche Applied Science). The data was calculated relative to the control siRNAs after normalization to *GAPDH* expression. The following primers were used: *TAF4* forward 5′-CTGCCACGGAAA CTATGGAA-3′, reverse 5′-GCTAACGCGGTCCTGTAA AG-3′; *CDK2* forward 5′-CCTCTGCTCTCACTGGCATT -3′, reverse 5′-GGACTCCAAAAGCTCTGGCTA-3′; *CDKN1A* forward 5′-CACTGTCTTGTACCCTTGTGC-3′, reverse 5′-GGATTAGGGCTTCCTCTTGG-3′; *TP53* forward 5′-TGGAGGAGCCGCAGTCAGATCC-3′, reverse 5′-GGATTAGGGCTTCCTCTTGG-3′; *SOX2* forward 5′-GAAGAAGGATAAGTACACGCTGC-3′, reverse 5′-GTTCATGTGCGCGTAACTGT-3′; *NF-M* forward 5′-GAGTGGTTCAAATGCCGCTAC-3′, reverse 5′-CTCT AGCTCGATGCTCTTGGA-3′; *NTRK2* forward 5′-TGAT GATGACTCTGCCAGCCC-3′, reverse 5′-TTGAGCTG ACTGTTGGTGATGCCA-3′; *GAD2* forward 5′-GGCT TTTGGTCTTTCGGGTC-3′, reverse 5′-TTCTCGGCGT CTCCGTAGAG-3′; *GFAP* forward 5′-ATCGAGAAGG TTCGCTTCCTG-3′, reverse 5′-TGTTGGCGGTGAGT TGATCG-3′; *SYP* forward 5′-AGTTGGGGACTACTCC TCGTC-3′, reverse 5′-GGCCCTTTGTTATTCTCTCGGT A-3′; *DKK1* forward 5′-ATAGCACCTTGGATGGGTAT TCC-3′, reverse 5′-CACAGTCTGATGACCGGAGA-3′; *WNT5A* forward 5′-ATGGCTGGAAGTGCAATGTCT-3′, reverse 5′-ATACCTAGCGACCACCAAGAA-3′.

Immunoblotting

Upon RNAi, proliferating NHNP cells (48 h after siRNA treatment) and NHNPs induced to neuronal or glial differentiation for 5 days were lyzed and exposed to Western blotting as previously described (Kazantseva et al. 2013). The following primary antibodies were used: TAF4 (1:250, BD Biosciences, 612054), $TP53^{Ser15}$ (1:1,000, Cell Signaling, 9284), nestin (NES) (1:500, R&D Biosystems, MAB1259), GFAP (1:1,000, R&D Biosystems, MAB2594), TUJ1 (1:2500, Sigma-Aldrich Life Science, T2200), and GAPDH (1:10,000, Sigma-Aldrich Life Science, G8795).

Immunofluorescence

NHNP cells nucleofected with control or *TAF4* siRNAs were seeded on poly-D-lysine-coated glass slides at density 5×10^4 cell/cm^2 and were induced 24 h later towards neuronal or glial differentiation. After 4 days of differentiation, the cells were treated as previously described (Kazantseva et al. 2013) and stained using primary antibodies (GFAP (1:600), TUJ1 (1:600)), and Alexa Flour 546 or Alexa Flour 488-conjugated secondary antibodies (Molecular Probes, Invitrogen). Images were obtained using Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Inc., USA).

Statistical Analysis

Statistical analysis was performed using an unpaired Student's *t* test with a two-tailed *p* value. Differences were considered significant when the *p* value was less than 0.05.

Results

In previous work, we have described expression of alternative splice variants of *TAF4* in different tissues derived from human mesenchymal stem cells and demonstrated the role of hTAF4-TAFH activity in the lineage commitment decisions (Kazantseva et al. 2013). In order to evaluate the role of hTAF4-TAFH in neural development, we characterized the expression of *TAF4* alternatively spliced mRNAs encoding protein isoforms with altered hTAF4-TAFH co-activator domain in different regions of the human brain using RT-PCR followed by sequencing analysis. Our data revealed that *TAF4* alternative splice variants (ASVs) with in-frame deletions in the hTAF4-TAFH domain, as well as the canonical form of *TAF4*, were differentially expressed in various brain regions (Fig. 1a). For example, ASV *TAF4_v5* was distinctly expressed in colliculi while *TAF4_v4* was predominantly detected in the optic nerve, cerebellar white matter, ventral pons, cerebral pedunculi, substantia nigra, and nucleus ruber (Fig. 1a). Certain ASVs were expressed in a relatively invariant manner across brain sections, similarly to the pattern observed in human nonneuronal tissues (Kazantseva et al. 2013). Alternative splice variants *TAF4_v2* and *TAF4_v9* were widely expressed in different brain regions (Fig. 1a). Interestingly, ASV *TAF4_v2* encoding isoform lacking almost the entire hTAF4-TAFH domain was expressed at high levels in all analyzed brain sections.

Next, we studied the expression of TAF4 in NHNPs directed to differentiation towards glial and neuronal lineages. The levels of expression of the canonical form of TAF4 decreased significantly by day 7 upon neural differentiation of NHNPs as evidenced by Western blot analysis (Fig. 1b). Moreover, along with the downregulation of *TAF4_v1* expression, *TAF4* ASVs encoding isoforms with impaired hTAF4-TAFH domain emerged notably (Fig. 1b).

To examine the functional role of hTAF4-TAFH domain in neural differentiation, we transfected NHNPs with two different siRNAs targeting exons V and VI of *TAF4* encoding the functionally active hTAF4-TAFH domain. Successful silencing of *TAF4_v1* encoding the canonical form of TAF4 was observed with both *TAF4* siRNAs at 50-nM concentration as confirmed by RT-PCR and Western blot analyses (Fig. 1c, d). Concomitantly, we detected a selective switch from the expression of *TAF4_v1* to the expression of ASVs encoding the isoforms with impaired hTAF4-TAFH domain (in particular, *TAF4_v9* and *TAF4_v10* ASVs). Both, *TAF4_v9* and *TAF4_v10*, preserve ORF and have altered hTAF4-TAFH domains (Kazantseva et al. 2013). Expression of *TAF4_v2* ASV remained unaffected by *TAF4* siRNA treatments (data not shown).

Given that effects of TAF4 on differentiation of hMSCs were partly mediated by TP53 activation (Kazantseva et al. 2013), we analyzed the functional conservation of the role of TP53 in governing TAF4 activity in NHNPs. Increased levels of *TP53* mRNA expression accompanied by TP53 Ser15-phosphorylation were observed at 24 h post-siRNA treatment (Fig. 1d). Moreover, elevated levels of cell cycle regulators and direct targets of *TP53*, such as *CDKN1A* and *CDK2*, were detected in NHNPs treated with *TAF4* siRNAs, but not with control siRNAs at 6 h post-treatments. Altogether, these data indicate the conserved role of TP53 in mediating hTAF4-TAFH-dependent cell cycle arrest in NHNPs and hMSCs.

To demonstrate the role of hTAF4-TAFH activity in the process of differentiation of NHNPs, we analyzed neuronal and glial differentiation of cells transfected with *TAF4* or control siRNAs. Overall, NHNPs treated with *TAF4* siRNAs showed more spontaneous differentiation compared with control siRNA-treated cells. This was evidenced by a significant induction of the expression of a set of neural markers such as *NF-M*, *NTRK2*, and *GAD2* mRNAs in NHNPs at 24 h post-treatment (Fig. 1e). The levels of expression of *DKK1*, a WNT signaling inhibitor, and *WNT5A*, a ligand of the noncanonical WNT pathway, were notably increased in NHNPs post RNAi treatment. Similar phenomenon was observed in hMSCs (Kazantseva et al. 2013), suggesting a conserved switch from canonical to noncanonical WNT signaling mechanism in cells with declined hTAF4-TAFH activity. Furthermore, Western blot analysis confirmed significant downregulation of the expression of intermediate filament protein NES in NHNPs at 48 h post-treatment (Fig. 1f). Upon glial differentiation of *TAF4* siRNA-treated NHNPs, the level of *GFAP* mRNA was substantially elevated by 96 h as compared with control siRNA-treated cells (Fig. 1e). During neuronal differentiation of NHNPs, a significant increase in the expression of neuron-specific markers *NTRK2*, *SYP*, and *GAD2* was observed by 96 h following *TAF4* RNAi (Fig. 1e). Western blot confirmed enhanced expression of GFAP (~3-fold for both, *TAF4_ex5_*-siRNA and *TAF4_ex6_*siRNAs) and TUJ1 (>2-fold increase for both siRNAs) in NHNP-derived glial or neuronal cells at day 5 post-treatment, respectively (Fig. 1f). Immunofluorescence analysis of the expression of glial-specific GFAP and neuron-specific TUJ1 proteins demonstrated that both control and *TAF4* siRNA-treated NHNPs differentiated along glial and neuronal lineages (Fig. 1g). However, accelerated and more robust differentiation of *TAF4* siRNA-treated NHNPs towards glial and neuronal lineages was evidenced by earlier morphological changes not yet visible in control cells at the similar time point.

Consistent with these results, overexpression of TAF4 in NHNPs for 24 h, followed by neuronal differentiation for 4 days, led to increased expression of *SOX2* that is critical for maintenance of pluripotency. Marked downregulation of expression of genes associated with neuronal and glial differentiation, including *NTRK2*, *GAD2*, *SYP*, and *GFAP*, was detected (Fig. 1h).

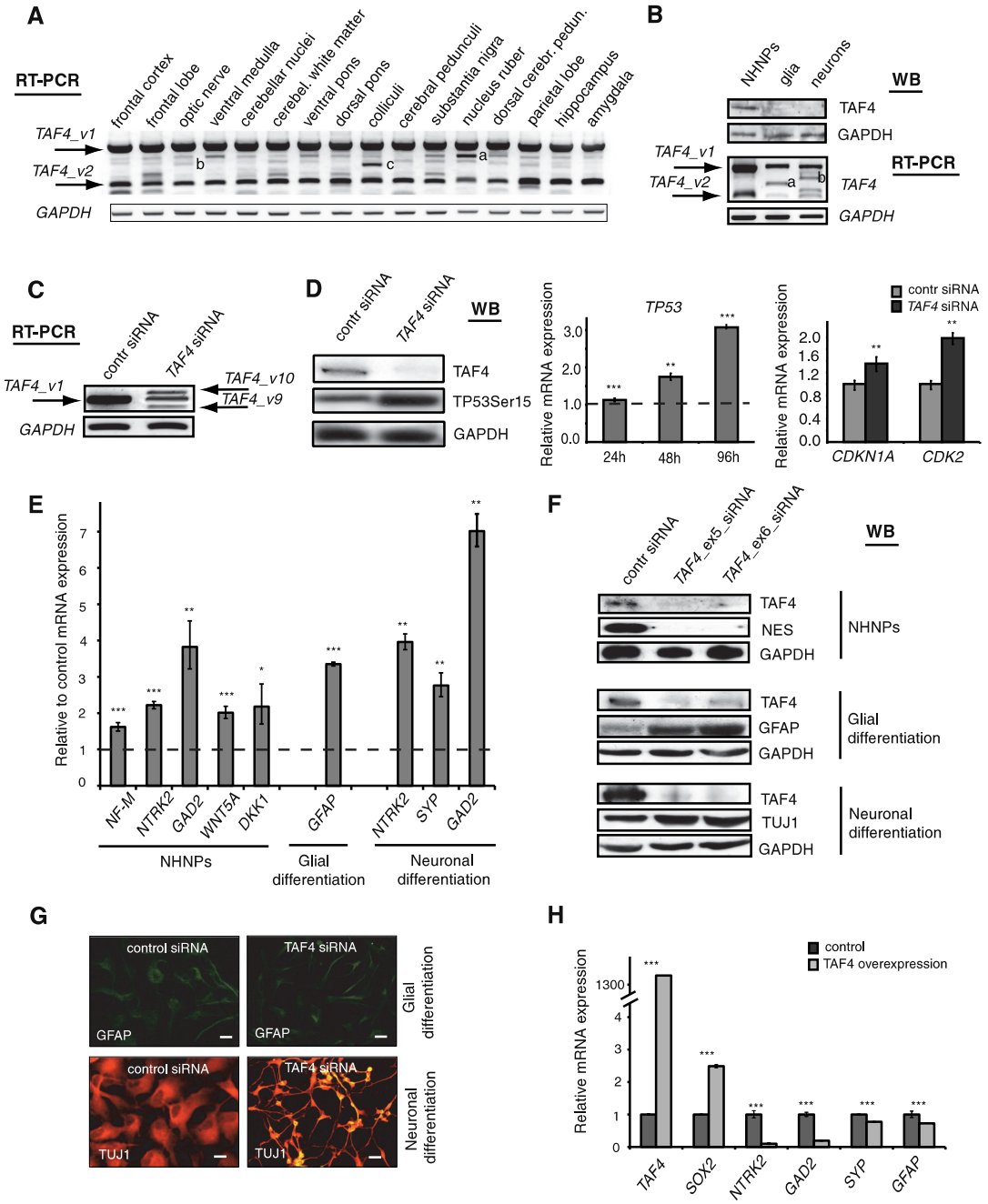
Altogether, these data argue that *TAF4* splice variants affect the balance between proliferation and differentiation. Suppression of hTAF4-TAFH activity and/or expression of *TAF4* splice variants encoding proteins with hTAF4-TAFH-impaired function accelerate neural differentiation of NHNP cells, while predominant expression of the canonical form of TAF4 protein keeps neural progenitor cells in an undifferentiated state. In this work, we provide the first evidence that TAF4 acts as a gatekeeper of early neural differentiation by inhibiting premature activation of TP53 and by switching between WNT pathways.

Discussion

In this study, we evaluated the role of hTAF4-TAFH coactivator binding domain of TAF4 in neurogenesis. We show that the majority of regional variations of alternative splicing events affect exons encoding for hTAF4-TAFH domain in the human brain. How exactly these ASVs contribute to the coactivator function of TAF4 remains largely unknown. *TAF4* ASVs can be translated to functional proteins or, alternatively, they can perform a regulatory function at the RNA level. Herein, we provide evidence that RNAi of hTAF4-TAFH results in premature neural differentiation and that high levels of TAF4 are needed to support stemness in neural progenitor cells. Our findings also show that abolishing of hTAF4-TAFH activity induces the activation of TP53 signaling and switches in WNT pathway leading to accelerated neurogenesis of neural progenitor cells.

Recent studies have revealed that only 6 out of 14 of the canonical TAFs are detected in human embryonic stem cells (hESCs), among which TAF4 is also absent in the TFIID complex of actively transcribed genes (Maston et al. 2012). Altered composition of TFIID complex is reported to result in the aberrant expression of pluripotency-related genes and induction of differentiation. Furthermore, a recent study demonstrated that TAF4 alone is sufficient for generation of induced pluripotent stem cells (iPSC) (Pim Pijnappel et al. 2013). In addition, earlier findings have demonstrated that addition or removal even one of the TAF subunits of TFIID complex induces differentiation (Jones 2007; Deato and Tjian 2008; Freiman 2009). Moreover, diversity of alternative splice variants has been found to be the highest in undifferentiated hESCs and drop significantly during differentiation (Wu et al. 2010). Present study is in good agreement with all these

Fig. 1 Disruption of hTAF4-TAFH domain activity by *TAF4* RNAi leads to enhanced neural differentiation of human neural progenitor cells. **a** Analysis of expression of *TAF4* ASVs in the regions of human brain was performed by RT-PCR using primers that amplify transcripts across exons IV to X. *Arrows* indicate the positions of *TAF4_v1* and *TAF4_v2* mRNAs encoding the canonical form of TAF4 and isoform with almost entire hTAF4-TAFH domain deleted. *Letters* indicate to (*a*) *TAF4_v9*, (*b*) *TAF4_v4*, and (*c*) *TAF4_v5* mRNAs with partially deleted sequences encoding for the hTAF4-TAFH domain. Equal loading was verified by analysis of *GAPDH* expression. ASVs with frame-shifts in ORFs are not indicated. **b** Data of Western blot and RT-PCR analyses of NHNPs directed towards glial and neuronal differentiation for 7 days indicate the absence of expression of canonical TAF4_v1 protein (*upper*) and increased expression of *TAF4* ASVs encoding isoforms with impaired hTAF4-TAFH domain, where *letters* indicate to alternative transcripts (*a*) *TAF4_v5*, (*b*) *TAF4_v4*. **c** RT-PCR analysis of expression of *TAF4_v1*, *TAF4_v9*, and *TAF4_v10* in control or *TAF4* siRNA transfected NHNPs at day 3 post-transfection. *TAF4_v9* and *TAF4_v10* encode proteins with altered hTAF4-TAFH domains. **d** Impairment of hTAF4-TAFH activity by RNAi leads to TP53 activation and the expression of cell cycle regulators. NHNPs were transfected with 50 nM of the control or *TAF4* specific siRNAs. The results of Western blot analysis show silenced expression of the canonical form of TAF4 and a significant activation of TP53Ser15 in response to *TAF4* siRNA treatments. NHNPs were analyzed at 24 h upon *TAF4* siRNA transfection (*left*). Dynamic induction of *TP53* expression in NHNPs analyzed by RT-qPCR in response to *TAF4* siRNA treatments. Data are presented as changes in expression relative to control siRNA treatments (*middle*). RT-qPCR analysis of relative expression of cell cycle regulators cyclin-dependent kinase inhibitor 1A (*CDKN1A*) and cyclin-dependent kinase 2 (*CDK2*) of NHNPs cells using *TAF4* siRNA as compared with expression in cells transfected with control siRNAs at 6 h post-treatments (*right*). Statistically significant differences are indicated (Student's *t* test; $***P<0.001$, $**P<0.01$). **e** RT-qPCR analysis of the expression of glial and neuronal markers in NHNPs at 24 h post-siRNA transfection (*NHNPs*) and 96 h upon glial and neuronal differentiation of siRNA-treated NHNPs. The fold of mRNA induction of *TAF4* siRNA-treated samples represents expression values normalized to the expression of the control siRNA transfected samples. Statistically significant differences of three independent experiments are indicated (Student's *t* test; $***P<0.001$, $**P<0.01$, $*P<0.05$). **f** Western blot analysis evidenced the expression of lineage-specific marker genes in *TAF4* siRNA-treated cells as compared with the control siRNA-treated NHNPs at different time points. NES expression was analyzed 48 h after *TAF4* siRNA treatment and prior to stimulation of differentiation in respective media. Glial and neuronal differentiated NHNPs were analyzed at day 5 after siRNA transfection and following the induction of differentiation. Precocious expression of GFAP and TUJ1 in the protein lysates of TAF4 RNAi exposed NHNPs indicated to the accelerated differentiation processes. Effective silencing and equal loading were verified analysis of TAF4 and GAPDH expression, respectively. **g** Immunofluorescence analysis of *TAF4* or control siRNA-treated NHNPs. siRNAs were transfected into the NHNPs 24 h prior to stimulation of differentiation and cells were differentiated subsequently for 4 days. Glial fibrillary acidic protein (*GFAP*) staining was used for identification of glial, and neuronal class III (*TUJ1*) for neuronal cells. *Scale bars*, 25 μm. **h** RT-qPCR analysis of the expression of neural markers in NHNPs transfected with TAF4 expression plasmid and subjected to neuronal differentiation on the following day after transfection and analyzed 4 days after stimulation of differentiation. The fold of mRNA induction of *TAF4* in transfected samples represents expression values normalized to the expression in the cell samples transfected with the control vector. Statistically significant differences are marked with $***P<0.001$



findings arguing that levels of expressed TAF4 isoforms are tightly regulated by alternative splicing in order to maintain cells in a more immature state.

Recent analysis of the human brain transcriptome during brain development linked neural function with discrete splicing patterns (Mazin et al. 2013). In general, neural-specific

splicing of transcriptional regulatory factors is widely described in development, neurodegenerative pathologies, and cancer. Neuron-specific splicing of neural repressor REST contributes to the control of neurogenesis and formation of neuroblastoma tumors (Palm et al. 1998, 1999; Raj et al. 2011). The N-terminally truncated isoform of BAF57, a component of SWI/SNF complex, is implicated in neurogenesis (Kazantseva et al. 2009); while the neuron-specific isoform of TAF1 could be related to progressive neurodegeneration in the striatum (Sako et al. 2011; Jambaldorj et al. 2012). Herein, we show that *TAF4* ASVs encoding proteins with altered hTAF4-TAFH domain structure are widely expressed across the adult human brain. As the hTAF4-TAFH co-activator domain interacts with a set of transcriptional regulators, it is evident that a modified domain cannot recognize its canonical ligands and becomes suited for noncanonical cellular functions. Similar regulation of the protein activity by alternative splicing is observed for polypyridine tract binding (PTB) protein (Boutz et al. 2007). General ubiquitously expressed isoform of PTB represses spliceosome, thereby altering its assembly and affecting splicing efficiency. In the brain, however, expression of neuron-specific form of PTB with low binding activity contributes to the high levels of alternative splicing events.

Present findings provide new evidence that hTAF4-TAFH domain of TAF4 adds to the control of neural differentiation and could be a valuable tool for studying global mechanisms of brain health and disease. We found that impairment of the hTAF4-TAFH activity of TAF4 is accompanied by accelerated neural differentiation of NHNPs. Targeting hTAF4-TAFH activity of TAF4 by RNAi resulted in a significant downregulation of nestin that is predominantly expressed in stem/progenitor cells and spontaneous upregulation of numerous neural markers. Furthermore, TAF4 RNAi-treated NHNPs showed increased expression of glial- or neuronal-specific genes analyzed at 96 h post-differentiation by RT-qPCR and at 5 days by Western blot confirming a more rapid conversion to neuronal and glial cells. These molecular alterations were accompanied with hastened changes in cellular morphology. In contrast, high levels of TAF4 supported the pluripotent state of NHNPs by preventing differentiation. Together, our data argue for the role of hTAF4-TAFH activity as critical for timely regulation of neural differentiation.

Here, we also show data of the involvement of TP53 and WNT signaling pathways in TAF4-mediated neural differentiation. Implication of TP53 in various developmental programs is well established (Stiewe 2007; Molchadsky et al. 2008). TP53 has been shown to control neuronal differentiation (Eizenberg et al. 1996). A genome-wide screening study has revealed that the WNT signaling pathway is one of the major targets of TP53 activity (Lee et al. 2010). Interestingly, in neural progenitors, both canonical and noncanonical WNT signaling has been found to control differentiation to various degrees in a stage-specific manner (Montcouquiol et al. 2006; Davis et al. 2008; Munji et al. 2011). Furthermore, the expression of DKK1, an inhibitor of the canonical WNT pathway, is controlled by TP53 activity (Wang et al. 2000). Overall, our findings are well supported by these studies and strengthen the notion that *TAF4* ASVs encoding TAF4 isoforms with altered hTAF4-TAFH activity control neurogenesis through synergistic activation of TP53 and WNT pathways, although the detailed mechanisms have yet to be established.

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Conflict of interest The authors declare no conflicts of interest.

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Review

Diversity in TAF Proteomics: Consequences for Cellular Differentiation and Migration

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Abstract: Development is a highly controlled process of cell proliferation and differentiation driven by mechanisms of dynamic gene regulation. Specific DNA binding factors for establishing cell- and tissue-specific transcriptional programs have been characterised in different cell and animal models. However, much less is known about the role of "core transcription machinery" during cell differentiation, given that general transcription factors and their spatiotemporally patterned activity govern different aspects of cell function. In this review, we focus on the role of TATA-box associated factor 4 (TAF4) and its functional isoforms generated by alternative splicing in controlling lineage-specific differentiation of normal mesenchymal stem cells and cancer stem cells. In the light of our recent findings, induction, control and maintenance of cell differentiation status implies diversification of the transcription initiation apparatus orchestrated by alternative splicing.

Keywords: TAF4; cell-specific transcription; alternative splicing; cancer stem cells

1. Introduction

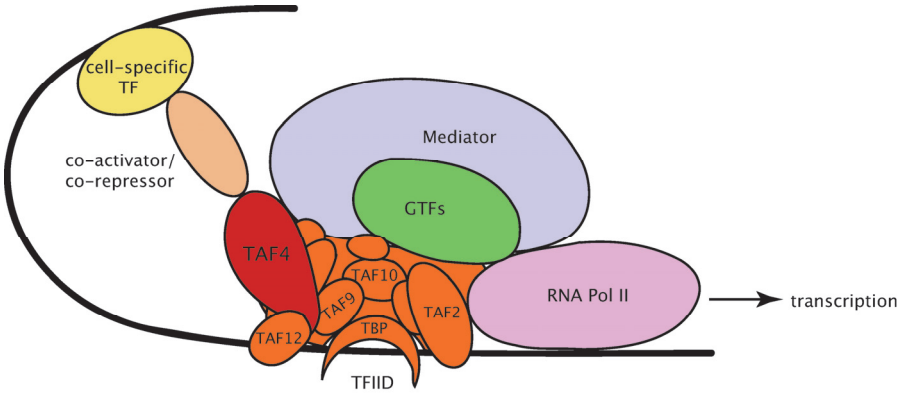
Cell-specific differentiation for tissue reconstruction is an extensively studied area of research. Common mechanisms of body development by consequent induction of appropriate paracrine and transcription factors are well characterized. Although tissue- and cell-specific transcription factors are widely studied, the role of general transcription machinery in tissue development and homeostasis is not well understood. Next to the spatiotemporal activity of general transcription factors that governs cell differentiation processes, another core process with significant influence on cell-fate decision is alternative splicing. Besides generating protein diversity, coordinated regulation of a myriad of developmental mechanisms in the body is accomplished through the diverse array of alternative protein isoforms and non-coding RNAs. Herein, we focus on reviewing the role and functional changes in the core transcription factor complex IID (TFIID) in controlling pluripotency and lineage-specific differentiation of normal and cancer stem cells. Being at the top of a hierarchical structure that governs vital cell functions, core transcription complex subunits, including TAF4, present a valuable target in understanding cell differentiation and tissue formation, and for advancing tissue-engineering approaches of the clinic.

2. The Diversity of Transcription Factor Complex IID (TFIID) Complex Subunits in Organism Development

Initiation of transcription is the first critical step in the regulation of gene expression. It requires simultaneous operation of a large cohort of transcriptional players—co-activators, trans-activators and components of the preinitiation complex (PIC), participating in the core promoter recognition [1,2] (Figure 1). However, the general transcription machinery that consists of RNA Pol II and the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH is necessary and sufficient for basal (core-promoter mediated) transcription.

TFIID is one of the several general transcription factors that compose PIC. TFIID is a large multi-subunit complex that together with TBP participates in the core promoter recognition [3]. TBP-associated factors (TAFs) were biochemically identified as stably associated with TBP components and originally named according to their electrophoretic mobility values [4]. Besides TBP, TFIID contains up to 14 different TAF subunits. The core composition of TFIID is well conserved from yeast to mammals. Interestingly, in addition to TBP, at least two TAFs bind to core promoters in a sequence-dependent manner [5]. Other components of the TFIID complex recognise multiple regulatory *cis*-elements in various combinations, as well as interact with modified nucleosomes and have enzymatic activities [6,7].

Figure 1. Multi-subunit tissue-specific RNA Pol II preinitiation complex (PIC) at the site of transcription. The main components of basal transcription preinitiation machinery (RNA Pol II (purple), GTFs (green), and Mediator (blue)) are presented. The tentative placement of some TBP-associated factors (TAF subunits) in the canonical TFIID complex (orange) is shown. TAF4 is specifically highlighted in brown. The co-activators/co-repressors (light brown) bridge upstream DNA-bound cell-specific transcription factors (yellow) with transcription machinery. It should be noted that various PIC complexes with different subunit compositions and sequence variation (isoforms) might be present, thus further diversifying PIC architecture beyond the example shown in this figure. TF, transcription factor; TBP, TATA box-binding protein; GTFs, general transcription factors.



Different genes utilize unique composition and structural architecture of TBP and TAF subunits, suggesting mechanisms of cell-type-specific regulation of basal transcription factors [8]. The universality of TBP was questioned when TBP-related factors, including TRF1, TRF2 and TRF3, were discovered [9]. TRF1, which is highly homologous to TBP, represents a subunit of an alternative core promoter complex that in *Drosophila* directs promoter-selective transcription via different polymerases in a subset of tissues, including nervous system and gonads [10,11]. TRF2, although broadly conserved from *Caenorhabditis elegans* to human, does not bind to TATA box sequences and is unable to recruit canonical TFIID complexes [12,13]. TRF2 is required for early embryonic development [14] and is highly expressed in mouse testis [15]. These observations imply that only a limited set of core promoter recognition complex subunits, such as TBP and TRF2, is needed to produce highly varied expression patterns. Although TRF3 (TBP2) is widely expressed in adult mammalian tissues, its role is crucial for oogenesis and in early embryonic development [16]. Developmental studies of *Xenopus* have demonstrated that TRF3 can partially rescue loss of TBP, suggesting different activation mechanisms rather than recruitment of specific transcription complexes [17]. TRF3 is essential for the initiation of hematopoiesis [18] and together with TAF3 is required for differentiation of myotubes [19].

Additionally to core TAFs, TAF paralogs are expressed in different cells and tissues [20]. For example, TAF4b [21] together with TAF7L [22] were initially discovered as B-cell-specific, and are involved in oogenesis and spermatogenesis and share similarities in domain structure with respective TAFs. Association of TAF7L and TBP governs adipogenesis through the binding with PPARG-RXR cofactors and directing adipocyte-specific differentiation [23]. Five homologues of canonical TFIID

subunits are expressed during spermatogenesis in *Drosophila*. Namely, the Cannonball, homologue of TAF5; No hitter, homologue of TAF4; Meiosis I arrest, homologue of TAF6; Spermatocyte arrest, homologue of TAF8; and Ryan express, homologue of TAF12 [24]. As a rule, up-regulation of expression of the paralog leads to the dynamic down-regulation of its core partner with accompanying changes in TAF sub-complex structure and composition.

Despite emerging data on the diverse composition of general transcription complexes and their heterogeneity of components during development, very little is known of the orchestration of the basal transcriptional activity in tissue-specific differentiation.

3. The Dynamics of TFIID Complex Components in Development and Differentiation

Given that the diversity of TFIID complexes in development is vast, their composition and functional dynamics at different stages of development are poorly understood.

At the earliest stage of vertebrate development, in the fertilised egg, two dynamic processes are highly crucial: zygotic gene activation and degradation of maternal mRNAs. Both of these events are regulated by the TBP activity [25]. Up-regulation of nuclear TBP levels promotes zygotic gene activation and the ratio of nuclear to cytoplasmic TBP protein regulates gradual degradation of cytoplasmic maternal mRNAs. Basal transcription complex dynamics in zygotic gene activation of *C. elegans* has been characterised for TAF5, TAF10 and TAF11 subunits [26]. In two-cell stage, TAF5 was identified to be vital for the open-complex formation, whereas TAF10 and TAF11 were detected in four-cell-stage nuclei with no impact on RNA Pol II activation. Similarly, in zebrafish embryos, both TBP and TAFs are highly expressed during early phases of gastrulation and their levels drop sharply at later stages of development [27].

Interaction of the intact basal transcription machinery with a set of tissue-specific transcription factors was previously suggested to be required for embryonic development and differentiation. However, more recent publications argue that lineage-specific differentiation involves the selective loss of some of the common RNA Pol II core complex subunits [9]. In addition, expression of different TFIID subunits is comparatively lower in non-differentiated cells as compared to fast proliferating cells [20]. Whether TFIID subunits are actively degraded or replaced by paralogs is currently not clear. Individual TAFs interact with upstream transcription factors and require specific core complex-specific partners for their functional activity. Consistent with this, canonical TFIID complexes do not contribute to transcription of the majority of genes in terminally differentiated hepatocytes [28]. TRF3–TAF3 complexes drive hematopoiesis and myogenic differentiation [18,29], and are essential for endodermal lineage commitment [30]; TAF4 controls ATRA-dependent differentiation [31], and TAF8 is involved in adipogenesis [32]. On the other hand, different transcription initiation mechanisms co-exist in cells during differentiation. Expression of some genes requires most of the canonical TFIID complex subunits, while others are transcribed by subsets of TFIID components. For example, expression of TAF8 is not detected in preadipocytes but is up-regulated during adipogenic differentiation, when the expression of other TAFs is down-regulated [32]. TAF10 is required for normal liver development [33]. Reduced expression of Taf1 and Taf4b affects proliferation of mouse embryonic maxillary mesenchymal cells and causes aberrant bone formation [34].

Germ cells are the best-characterised model of the transcription machinery adaptation during differentiation [35,36]. So far, the most diverse set of TBP and TAF paralogs is found in germ cells, supporting the concept of specialised TFIID complexes that are distinct from the canonical forms. For example TAF4b, the first identified tissue-specific TAF, is important for ovarian follicle development and function [37]. Male germ cell-specific TAF7L governs male fertility [38]. Expression of meiotic genes in gametes is controlled by TRF2 [39]. Oocyte-specific TRF3 replaces core TBP in these highly specialised cells, and its expression decreases during fertilisation, being substituted by TBP [40].

On the whole, very different models of transcription initiation operate during embryonic development and in the adult organism, supporting the concept of different transcription regulatory networks in proliferating and differentiating cells [41]. Identity of ESCs is governed by a set of sequence-specific pluripotent transcription factors, including OCT4, MYC, KLF4 and NANOG [42]. These factors control the activity of the basal transcription complexes via specific interactions with co-activators. Herein, TFIID activity contributes the most to the induction and maintenance of the highly plastic pluripotent state of ESCs [43]. Recent studies in mouse ESCs revealed preferential binding of TFIID to nucleosomes with the active epigenetic H3K4me3 and H3K14ac marks [44] and at genomic regions spanning transcription start sites of mouse ESCs [45]. Similar to early stages of development, in ESCs and pluripotent stem cells, active canonical TFIID complexes are required for cell function. The majority of TAFs are expressed in ESCs at high levels. TAF3 and TAF4 are specifically required for maintaining their pluripotency and function [30,46]. In contrast, different TFIID sub-complexes with redundant components are present in terminally differentiated cells, thereby reflecting the need of different cell types to respond to different external signals. High plasticity and adaptability of the core complex factors allow both highly specialised and broad initiation of transcription depending on the cellular context and developmental setup.

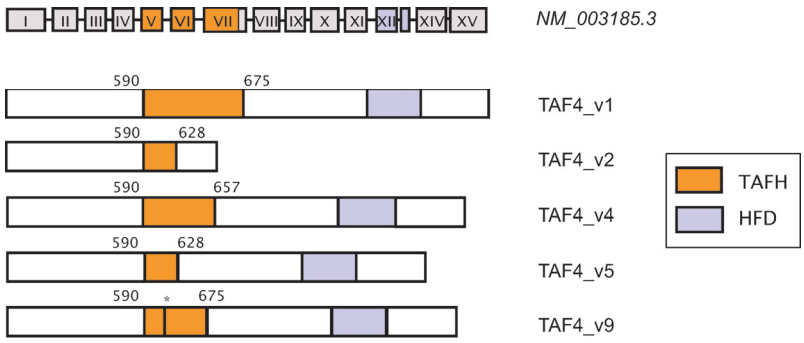
4. TATA Box-Binding Protein (TBP)-Associated Factor (TAF4): Structure, Function and Regulation

TAF4 is one of the largest and ubiquitously expressed subunits of the TFIID complex and is important for maintaining stability and integrity of the TFIID complex. Historically, TAF4 was the first TATA-binding associated factor with demonstrated co-activator function. Now, it is documented that TAF4 interacts with many activators, including Sp1 [47], CREB [48], NCoR [49], E-box transcription factors [50], and c-Jun [51]. TAF4 transcriptional activation is potentiated by the AF-2 motif of RARα, vitamin D3 and thyroid hormone receptors [52]. TAF4 interacts with HP1α and HP1γ, but not HP1β, further complicating its regulatory mechanisms and functions [53].

The molecular structure of TAF4 is remarkably conserved throughout evolution. Human TAF4 protein consists of an *N*-terminal metazoan-specific polyglutamine tract region, central co-activator binding TAFH domain (ETO, CRI), and *C*-terminal histone-fold domain (HFD, CRII) (Figure 2) [54]. TAF4 HFD is conserved from yeast to human and plays a critical structural role in the TFIID complex formation. By analogy with histone H2A and H2B, TAF4 interacts with TAF12 through structurally similar HFDs. Formed octamer-like sub-structures support the integration of TAF4 into TFIID and its binding to the core promoter regions in the chromatin. The length of the DNA sequence, which is recognised by the TAF4/TAF12 dimers, is about 70 bp and is half of the length of nucleosomal DNA. This suggests

the formation of hetero-complex structures, similar to the nucleosome complex assembly [55]. TAF4 HFDs are sufficient to nucleate the assembly of holo-TFIID complexes. In metazoans, TAF4–TAFH domain is highly conserved in all TAF4 and ETO family members [54]. Structural studies revealed that the α-helical folds of TAF4–TAFH domain forming a large hydrophobic groove are responsible for protein-protein interactions. Using the phage display screening method, the amino acid sequence that is targeting TAF4–TAFH binding surface was determined as DΨΨζζΨΦ (where Ψ represents V, I, L, or M; ζ represents hydrophilic residues including N, Q, S, or T; and Φ represents V, I, L, F, W, Y, or M) and was found to be present in different transcriptional regulators [54]. Thus, potential TAF4–TAFH binding partners include PBX proteins, important in limb development and hematopoiesis; HCF-1 dependent ZF and LZIP activators participating in chromatin modification and cell proliferation; Mediator subunit MED23 involved in post-translational remodeling of chromatin substrates; and a number of histone deacetylases, demethylases and kinases regulating cell cycle. Different to ETO–TAFH, vertebrate TAF4–TAFH domains exhibit unique packing of 5 helices and as a result, present very flat and extended binding surfaces. This allows a much broader spectrum of interactions and thus serves as a platform for positive and negative transcription regulation. It is important to note here that the *TAF4* gene is duplicated in mammals and has an ovarian-specific paralogue *TAF4b* [37].

Figure 2. Alternative splicing of human *TAF4* (*NM_003185.3*) leads to a variety of protein isoforms. *TAF4* gene structure and major TAF4 isoforms with deletions in the co-activator-binding hTAF4–TAFH domain are outlined. Some isoforms of TAF4, for example TAF4 ASV_v2, differ in their *C*-terminus. Functionally important TAFH (hTAF4–TAFH, orange) and HF (Histone-fold, blue) domains are highlighted. Boundaries of hTAF4–TAFH domain are shown by amino acid residue numbers above the structures and correspond to their position in the canonical form of TAF4, where the * symbol represents deletion of the amino acids 628–658 in the TAFH domain.



The role of TAF4 in a range of cellular physiological processes and in somatic cell reprogramming has been extensively studied and identified. In various organisms, downregulation of TAF4 activity has dramatic effects on cell fate. RNAi-mediated knockout of *Taf4* in *Drosophila* considerably disturbs TFIID stability, suggesting that TAF4 is its key structural subunit [56]. In *C. elegans*, depletion of TAF4 is highly destructive with effects similar to RNA Pol II loss-of-function [57]. Reversible and controlled repression of TAF4 is one of the mechanisms that regulate cellular development and differentiation. In the early embryo of *C. elegans*, TAF4 interacts with OMA-1/2, proteins that are necessary for oocyte

maturation. This interferes with TAF4 and TAF12 dimer formation and ultimately results in TAF4 sequestration to the cytoplasm. During the early stages of germline blastomere development, this process represses transcription. However, the degradation of OMA proteins in cytoplasm releases TAF4, which binds TAF12 and the resultant TAF4/TAF12 heterodimers translocate to the nucleus to restore transcription [57]. In mouse, *Taf4* is not essential for cell viability, but its inactivation affects proliferation [58]. Notably, *Taf4*$^{-/-}$ fibroblasts contain intact TFIID and do not exhibit cell cycle arrest or apoptosis. Interestingly, *Taf4* inactivation is associated with alterations in cellular morphology, serum-independent autocrine growth and deregulation of more than 1000 genes [58]. These changes in gene expression together with suppression of serum-independent growth are restored by re-expression of protein isoforms containing the CRII domain. *Taf4* depletion leads to high expression of TGFβ thereby enhancing SMAD (Sma and Mad Related Family) signalling by the positive feedback loop. Loss of *Taf4* further induces the expression of matrix metalloproteases, CTGF and OPN, which are important regulators of metastasis, thereby contributing to the oncogenic functions of TAF4. Ectopic expression of truncated forms of TAF4, containing TAFH but not CRII domains, yielded in accelerated cell growth. In contrast, cells expressing TAF4 CRII alone display slowed rate of growth [59]. The levels of TAF4 expression have been shown to control cell differentiation. Targeted proteolysis of Taf4 is observed in differentiating mouse F9 embryonal carcinoma cells [60,61], and during differentiation of C2C12 myoblasts into myotubes [62,63]. In contrast, enhanced expression of Taf4 impairs endodermal differentiation, whereas enhanced expression of CRII domains had no effects on this differentiation process in F9 cells [31]. These results showed for the first time that regulated degradation of TAF4 is required for differentiation into select cell lineages.

TAF4 activity is vital for different cellular physiological processes. TAF4 was demonstrated to act as a co-factor of retinoic acid receptors, and that its CRII domain alone is sufficient to mediate CREB and RAR activity. In a case of CRII deletion, it has been shown that ATRA can signal via TP53 to exert anti-apoptotic effects [31]. In addition, TAFH was identified as a domain targeted by Pygopus that promotes WG/WNT target gene transcription throughout *Drosophila* development [64,65]. Selective loss of *Taf4* in the mouse fetal epidermis results in aberrant skin appearance and histology, enhanced water loss and early post-natal death, suggesting defective skin barrier function [59]. In adult mouse epidermis, Taf4 participates in the normal hair cycle, as Taf4 deficiency results in fur loss. In addition, Taf4 deficit induces epidermal hyperplasia and aberrant differentiation of mouse adult basal keratinocytes. Moreover, Taf4 inactivation significantly alters cell adhesion, cell communication and induces the expression of markers correlating with oncogenic transformation. These changes stimulate tumour formation by enhancing malignant transformation. Upon histological analysis, the tumour cells exhibit melanocyte-like phenotype with high expression of genes involved in melanocyte signalling. However, it is worth noting that a set of genes affected by the loss of *Taf4* in mouse keratinocytes is different from that seen in the embryonic fibroblasts or fetal epidermis, suggesting involvement of different regulatory pathways. For example, in fibroblasts, the TGFβ pathway is activated, whereas in keratinocytes the EGF signalling is enhanced. Other studies connect *Taf4* inactivation in MEFs with the formation of fibrospheres and activated expression of pro-oncogenic Collagen 6A3 [66]. The mechanism involves repression of Hippo signaling and activation of the WNT pathway. Interestingly, treatment of MEFs by ATRA restores TAF4-abolished effects.

The most recent studies have focused on the function of TFIID, and TAF4 in particular, in reprogramming of somatic cells and ESCs. ChIP sequencing data revealed the binding of Nanog and Oct4 to the regulatory region of mouse *Taf4* gene [67], which acted as an ESC-specific enhancer. In MEFs and adult human fibroblasts, exclusion of TAF4 from the TFIID complex inhibited reprogramming of the somatic cells while TAF4 over-expression facilitated iPSC formation [46]. These findings evidence a positive feedback circuit between TAF4 and the pluripotency factors. Namely, enforced expression of TAF4 activates expression of the pluripotency factors, which in turn enable to maintain high levels of TAF4 expression.

Thus implicated in the majority of vital cellular processes, TAF4 as the component of the general transcriptional machinery is a valuable target for controlling cell functions.

5. Coordinated Switching of *TAF4* Alternative Splice Variants Controls Differentiation and Migration of Normal Progenitors and Cancer Stem Cells

Alternative splicing plays a key role in generating complex proteomes, breaking the "one gene, one protein" rule. Existence of multiple mRNA variants for a single gene explains, at least in part, the complexity of some organisms like humans, having about 20,000 protein-coding genes in their genome [68]. Moreover, alternative splicing enables quantitative gene control through regulation of various regulatory RNAs and by targeting RNAs to non-sense-mediated decay. Genome-wide analysis has revealed differential expression of alternative spliced mRNAs in various tissues and cell types [69,70].

Consistent with the notion that alternative splicing should avoid destruction of the protein domains, it targets mostly the areas of structure with minimally exposed hydrophobic surfaces and with high intrinsic disorder [71,72]. Removal of protein–protein interaction domains by alternative splicing affects drastically protein function. However, changed by alternative splicing protein–protein interaction domains are very common to regulators of transcription [73,74]. Ankyrin repeat, DNA-binding zinc finger, homeobox, and KRAB [75] domains of transcriptional factors are frequent targets of alternative splicing modification.

To date, a close link between transcription and splicing has been shown [76]. Transcription factors such as TAT-SF1, CA150, SKIP and co-activator PGC-1 are present in the spliceosome and perform dual functions in transcription and splicing. Thus, it is highly conceivable that both processes are simultaneously coupled together in space and time [77]. Consistent with this, the *C*-terminal domain of RNA Pol II directly participates in exon recognition [78]; differences in promoter structure recognition are often associated with differences in alternative splicing of pre-mRNA [79]; some transcriptional co-activators and co-regulators modulate alternative splicing, sometimes in a synergetic manner [80,81].

One of the major challenges today is studying the role of specific splice variants in the cell context. It has been shown that siRNA targeting of intron or exon sequences near the alternative-splicing sites affects the splicing process of these sequences [82]. Among protein isoforms whose function is studied by using this technique are adapter protein ShcA [83], spleen tyrosine kinase [84], and pyruvate kinase M1 and M2 [85].

Analyses of the alternative splicing of *TAF4* in various mouse and human cells and tissues identified multiple and complex splicing patterns [86,87]. In mouse, five alternative isoforms with deletions in the functional domains result in dominant negative effects in nuclear receptor-mediated

transcriptional activation [86]. Complex patterns of *TAF4* alternative splicing occur in different human tissues [87]. Although, a significant number of *TAF4* alternatively spliced mRNAs contain a premature termination codon, indicating that these splice transcripts are subjected to the nonsense-mediated RNA decay, others preserve the open reading frame (ORF) and could be considered as possessing some functional properties in the cell. Depending on the cell type, there is a different balance of expression between full-length *TAF4* and the rest of the alternative splice variants (ASVs). Some of the ASVs are invariantly expressed across tissues; others exhibit patterns of cell type-specific expression. Interestingly, the dominant expression of canonical full-length form of TAF4 is observed mainly in the cells with stem cells characteristics [86–88], while differentiated cells demonstrate multiple patterns of different combinations of *TAF4* ASVs. The most prone to alternative splicing exons of *TAF4* are those encoding the functional co-activator-binding hTAF4–TAFH domain (Figure 2). Since this domain is involved in the cooperation with a set of transcription activators like E-box proteins, WNT signalling mediating Pygopus, and others, it is likely that the structure of hTAF4–TAFH, which is cell-specifically targeted by alternative splicing, affects its interaction properties and dictates further cell development. As mentioned above, TAF4 is one of the structural components of TFIID complex and affects its stability. Therefore, alternative splicing of *TAF4* is most likely to have strong impacts on the integrity and functional activity of TFIID as well as on the core transcription machinery as a whole. Consistent with this, some of its alternative transcripts generate alternative protein isoforms, which differ from canonical TAF4 protein functions.

Our recent data demonstrate that *TAF4* RNAi leads to the appearance of new ASVs with serious effects on cell differentiation [87,88]. Namely, in human adipose-derived mesenchymal stem cells, silencing of canonical TAF4 activity and appearance of new hTAF4–TAFH-defected ASVs contributes to the repression of adipogenic and ostegenic programs, while promoting chondrogenesis [87]. Similarly, high expression of full-length *TAF4* is necessary for maintenance of multipotency in normal human neural progenitors but is dispensable for transcription in differentiated cells, as *TAF4* RNAi induces expression of ASVs and stimulates neuronal differentiation [88]. The exact mechanisms are currently unclear, although, our initial findings revealed the involvement of WNT and p53 signalling pathways. Our data showed the switching from canonical to non-canonical WNT pathways in response to *TAF4* RNAi [87]. It is interesting to note that other studies have found that suppression of canonical WNT signalling supports chondrogenesis, contributes to cell migration [89,90], and affects bone formation and limb development [91]. Thus, the switch from canonical to non-canonical WNT signalling could be one of the regulative mechanisms in TAF4 activity-deficient progenitor cells. p53 signalling appears also to play a role in differentiation of TAF4-deficient mesenchymal stem cells. Several recent studies link TP53 activity to cell differentiation [92] in addition to its well-established role in apoptosis. Interestingly, in TAF4-depleted mesenchymal stem cells (MSCs), increased levels of TP53 induce cell cycle arrest without any signs of apoptosis or cell senescence [87].

Adding support to the hypothesis that sustained expression of TAF4 impairs cell differentiation, *TAF4* RNAi induced spontaneous differentiation of facial dermal fibroblasts into melanocyte-like cells [93]. Phenotypic features of melanocyte-specific transformation of TAF4-deficient fibroblasts included darkening of the cell pellets due to melanin secretion and high levels of expression of master of melanogenic transcription factor MITF. This molecular transformation was accompanied by events reminiscent of the epithelial-to-mesenchymal transition, as *TAF4* silencing inversely correlated with

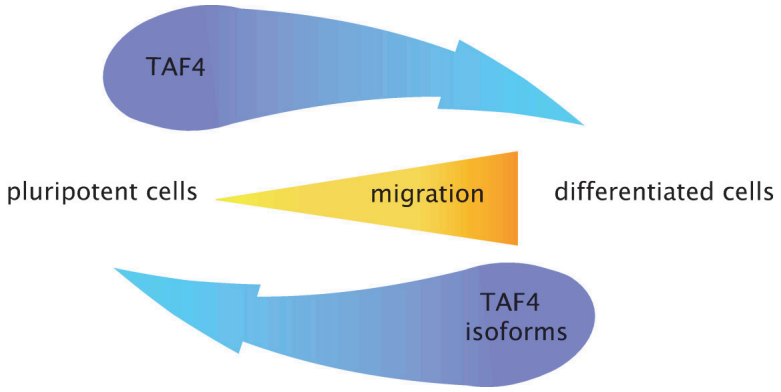
cadherin switch: down-regulation of TAF4 in the dermal fibroblasts suppressed *E*-cadherin and supported *N*-cadherin expression. It is known from other data that cadherin switch contributes to cancer progression and emergence of cells with stem-like characteristics [94,95].

Cells that are prone to multilineage differentiation, such as fibroblasts and mesenchymal stem cells, express canonical TAF4 at low levels as compared with wide expression of alternative splice variants encoding TAF4 isoforms. At the same time, cells with high pluripotent potential express elevated levels of canonical TAF4. In contrast, cancer cells, such as melanoma, express both canonical and other isoforms of TAF4 at high levels, thereby raising the question of the differentiation potential of tumour cells. Overall, highly plastic and heterogeneous melanoma cells fit well to the cancer stem cell model. Melanoma cells express pluripotent and differentiation-associated genes and differentiate into a wide range of cell types [96–98]. Altogether, our data using *TAF4* gain- and loss-of function studies allow the conclusion that melanomas have properties of stem cells and exhibit multilineage differentiation potential [93]. Through regulation of the *TAF4* ASVs expression balance in melanoma, it is possible to get melanoma populations with strong multipotent properties or drive cells to differentiate. Upon *TAF4* RNAi, a change in expression from canonical to alternative TAF4 isoforms with disturbed hTAF4–TAFH activity is observed, whereas removal of hTAF4–TAFH activity accelerates differentiation of melanoma cells along chondrogenic, adipogenic and neural lineages. The accelerated differentiation is accompanied with the down-regulated expression of melanoma-specific genes and cellular proliferation. In contrast, ectopic expression of the canonical TAF4 in melanoma cells leads to the up-regulation of pluripotency markers KLF4, OCT4 and NANOG [93]. Thus, pluripotency and differentiation status of normal and cancer cells can be controlled by *TAF4* ASV expression.

Targeting the self-renewal and differentiation potential of stem cells for clinical use is worthless if the migration of cells to target tissues cannot be appropriately controlled. Migration of stem cells to different organs and target niches requires active guiding, a process termed homing. Homing is necessary for tissue transplantation and for seeding stem progenitors during development. Being better understood and studied for hematopoietic stem cells, it is also applicable to mesenchymal and cancer stem cells [99,100]. Furthermore, considering that cancer is often a stem cell-retaining disease, it is important to understand the different features of homing and migration of cancer and normal stem cells in order to better control tumour progression. However, the molecular mechanisms underlying the motility potential of stem/progenitor cells are not well studied.

Our recent data demonstrate that reduced expression of full-length *TAF4* by RNAi leads to the enhanced motility of normal dermal fibroblasts and malignant melanoma cells [93]. In contrast, transient over-expression of canonical TAF4 diminishes the invasion potential of the melanoma cells. Observed changes were accompanied by molecular switches in E/N cadherin and matrix metallopeptidases expression. The regulated expression of canonical and other TAF4 isoforms reveals a remarkable conservation in different cell systems, and supports cell state transitions (between pluripotency and differentiation) and migration (Figure 3).

Figure 3. A model describing the cross-talk between canonical TAF4 and its alternative isoforms in pluripotent (stem cells) and differentiated cells, and their relation to migration. High levels of canonical TAF4 (blue arrow from left to right) are a characteristic to normal stem cells and cancer cells. In contrast, differentiated cells maintain high expression of TAF4 isoforms with deletions in hTAF4–TAFH domain (blue arrow from right to left). Functional balance between canonical and alternative isoforms of TAF4 is crucial for cell migration. Consistent with this, cells with low expression of canonical TAF4 are prone to migration contrasting with low motility of cells with high levels of TAF4 activity.



Altogether, our studies identify TAF4 as a critical modulator of cell differentiation and metastatic spread of cancer. Loss of hTAF4–TAFH activity by regulated alternative splicing affects cell homeostasis of normal and cancer cells. Regulation of the equilibrium of *TAF4* alternative mRNAs encoding functionally different protein isoforms provides the possibility to direct cell status towards either pluripotency or differentiation. This finding is reinforced by the observation that motility of normal and malignant cells and the expression of full-length *TAF4* are inversely correlated, thereby combining the concepts of cell migration in development and disease progression.

6. Conclusions

Deeper understanding of the molecular mechanisms behind cellular differentiation is extremely important for the development of new and improved cellular therapies. One of the novel approaches could include modulation of the composition and activity of basal transcription complex factors, for example, utilizing *Taf4* RNAi to enhance chondrogenic differentiation. This strategy has two significant benefits: suppression of TAF4 canonical activity accelerates chondrogenic differentiation and enhances motility of the mesenchymal progenitors, thereby directing modified cell pools to the distant target sites throughout the body.

In summary, the detailed understanding of TAF4 proteomics in different cellular contexts, including tumour cells, will clearly be of immense benefit for future prospects of cell-directed therapeutics.

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Author Contributions

Jekaterina Kazantseva reviewed literature, generated figures and wrote the manuscript. Kaia Palm contributed to revising and editing.

Abbreviations

ATRA, all-trans retinoic acid; BAF45/BAF53, BRG1-Associated Factor 45/53; CA150, co-activator of 150 kDa; ch-ERG, chicken *Ets*-related transcription factor; CREB, cAMP response element-binding protein; CTGF, connective tissue growth factor; c-Jun, V-jun avian sarcoma virus 17 oncogene homolog; EGF, epidermal growth factor; ETO domain, 8;21 translocation domain; GTFs, general transcription factors; HCF-1, host cell factor 1; HP, heterochromatin protein; KLF4, Krüppel-like factor 4; KRAB, Krüppel associated box; lncRNA, long non-coding RNA; LZIP, leucine zipper protein; MEFs, mouse embryonic fibroblasts; MIA/CD-RAP, melanoma inhibiting activity/cartilage-derived retinoic acid-sensitive protein; MITF, microphthalmia-associated transcription factor; MYC, myelocytomatosis viral oncogene; NCoR, nuclear receptor co-repressor; OCT4, octamer-binding transcription factor 4; OMA-1/2, overlapping activity with M-AAA protease 1/2; OPN, osteopontin; PBAF, polybromo-associated BAF; PBX, pre-B cell leukemia transcription factor; PGC-1, peroxisome proliferator-activated receptor-gamma coactivator; PPARG, peroxisome proliferator-activated receptors; RARα, retinoic acid receptor-alpha; RXR, retinoid X receptor; ShcA, Src homology 2 domain containing transforming protein A; SKIP, Ski-interacting protein; SMAD, Sma and Mad Related Family; Sox9, SRY (sex determining region Y)-box 9; Sp1, specificity protein 1; SWI/SNF, SWItch/Sucrose NonFermentable; TAF, TATA box-binding protein (TBP)-associated factor; TAT-SF1, cofactor required for Tat activation of HIV-1 transcription; TBP, TATA-box protein; TGFβ, transforming growth factor beta; WG/WNT, wingless; ZF, zinc finger.

Conflicts of Interest

The authors declare no conflict of interest.

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PUBLICATION IV

Kazantseva, J., Sadam, H., Neuman, T., Palm, K. (2014) Differentiation and migration properties of dermal fibroblasts and melanoma cells under the control of alternative splicing of *TAF4*. *Manuscript*

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2. September 7-17, 2009, Island of Spetses, Greece. Spetsai Summer School "Proteins and their Networks – from specific to global analysis". Oral presentation "Splicing of TAF4 antagonizes Wnt-induced transcription."
3. May 16-19, 2006, GE Eurolab Munich, Germany. Ettan DIGE Training Course
4. January 11-14, 2007. Salk Institute, La Jolla, CA, USA. Symposium on Biological Complexity: Diseases of Transcription. Poster presentation "Novel neuronal specific BAF57 isoforms description."
5. September 12-16, 2005, Lomonosov Moscow State University, Moscow, Russia. Summer School Behavioral Neurogenetics
6. June 2005, Tallinn, Estonia. IBRO Summer School in Neurobiology
7. August 25 - September 6, 2003, Institute of Biotechnology, Vilnius, Lithuania. HHMI practical course "Molecular Interactions of Proteins and DNA."

8. August 26-31, 2002, Tartu, Estonia. Practical course "Application of tagging techniques and alphavirus expression for functional analysis of proteins."

Publications:

Kazantseva, J., Palm, K. (2014) Diversity in TAF proteomics: consequences for cellular differentiation and migration. *Review*. Int. J. Mol. Sci. *15*(9), 16680-16697

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Intellectual property:

CANCER RELATED ISOFORMS OF COMPONENTS OF TRANSCRIPTION FACTOR COMPLEXES AS BIOMARKERS AND DRUG TARGETS; Owner: Oncotx, Inc; Authors: **J. Kazantseva**, A. Kazantseva; Patent number US 2010/0087376 A1.

MALDI MS-based high-throughput screening method for substances inhibiting aggregation of Alzheimer's A peptides; Owner: Tallinn University of Technology; Authors: P. Palumaa, M. Tuuling, O. Blaževitš, **J. Kazantseva**, K. Zovo, I. Šabarova; Patent number PCT/EE2008/000024.

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Hariduskäik:

2001-2006 Tallinna Tehnikaülikool, doktoriõpe geenitehnoloogia erialal
1995-1997 Lomonosovi Moskva Peenkeemiatehnoloogia Instituut, Venemaa, MSc diplom *cum laude* geeni- ja biotehnoloogias
1991-1995 Lomonosovi Moskva Peenkeemiatehnoloogia Instituut, Venemaa, BSc diplom *cum laude* biotehnoloogias
1981-1991 Tallinna 64. Keskkool, kuldmedal

Keelteoskus:

Keel	Tase
Vene	emakeel
Eesti	kõrgtase
Inglise	kõrgtase
Prantsuse	algtase

Erialane töökogemus:

Jan 2013 -	Cellin Technologies OÜ, proteoomika teadur
Jan 2012 – Dec 2012	VTAK, Protobios OÜ, teadur
Jan 2010 – Dec 2011	Cellin Technologies OÜ, teadur
Nov 2005 – Jan 2010	FibtoTx OÜ, teadur
Sept 2005 – May 2007	VTAK, Protobios OÜ, teadur
Sept 2002 – Dec 2009	Tallinna Tehnikaülikool, Geenitehnoloogia instituut, teadur
Sept 2001 – June 2002	Tallinna Kommertskolledž, lektor

Apr 2000 – Dec 2003	Eesti Tervisekaitseinspektsioon, Keskviroloogia Labor, ekspert
Sept 1995 – June 1997	Engelhardti Molekulaar Bioloogia Instituut, Moskva Teaduste Akadeemia, Venemaa, insener

Auhinnad ja stipendiumid:

2009: Poster-ettekande auhind Spetsai suvekoolis "Proteins and their Networks – from specific to global analysis"

1996-1997: Mendeleev stipendium, Lomonosovi Moskva Peenkeemiatehnoloogia Instituut

Kursused ja konverentsid:

1. September 15-18, 2013, Firenze, Itaalia. ISSCR regionaalne foorum "Stem cells in translation". Poster-ettekanne "hTAF4-TAFH activity of TAF4 inhibits chondrogenic differentiation of human mesenchymal stem cells and neural differentiation of human progenitor cells."
2. September 7-17, 2009, Spetses, Kreeka. Spetsai Suvekool "Proteins and their Networks – from specific to global analysis". Ettekanne "Splicing of TAF4 antagonizes Wnt-induced transcription."
3. Mai 16-19, 2006, GE Eurolab, München, Saksamaa. Ettan DIGE praktiline kursus
4. Jaanuar 11-14, 2007. Salk Institute, La Jolla, CA, USA. Sümpoosium "Biological Complexity: Diseases of Transcription". Poster-ettekanne "Novel neuronal specific BAF57 isoforms description."
5. September 12-16, 2005, Lomonosovi Moskva Riikliku Ülikool, Moskva, Venemaa. Käitumist neurogeneetika suvekool
6. Juuni 2005, Tallinn, Eesti. IBRO Neuroteaduste kursus
7. August 25 - September 6, 2003, Biotehnoloogia Instituut, Vilnius, Leedu. HHMI teoreetiline ja praktiline kursus "Molecular Interactions of Proteins and DNA."
8. August 26-31, 2002, Tartu, Eesti. Praktiline kursus "Application of tagging techniques and alphavirus expression for functional analysis of proteins."

Publikatsioonid:

Kazantseva, J., Palm, K. (2014) Diversity in TAF proteomics: consequences for cellular differentiation and migration. *Review*. Int. J. Mol. Sci. *15*(9), 16680-16697

Kazantseva, J., Tints, K., Neuman, T., Palm, K. (2014) TAF4 controls differentiation of human neural progenitor cells through hTAF4-TAFH activity. J. Mol. Neurosci. 2014 Apr 4

Kazantseva, J., Kivil, A., Tints, K., Kazantseva, A., Neuman, T., Palm, K. (2013) Alternative splicing targeting the hTAF4-TAFH domain of TAF4 represses proliferation and accelerates chondrogenic differentiation of human mesenchymal stem cells. PLoS One. 2013 Oct 2; 8(10):e74799

Kazantseva, A., Sepp, M., **Kazantseva, J**., Sadam, H., Pruunsild, P., Timmusk, T., Neuman, T., Palm, K. (2009) N-terminally truncated BAF57 isoforms contribute to the diversity of SWI/SNF complexes in neurons. Journal of Neurochemistry, 109, 807 - 818

Voronova, A., Meyer-Klaucke, W., Meyer, T., Rompel, A., Krebs, B., **Kazantseva, J**., Sillard, R., Palumaa, P. (2007) Oxidative switches in functioning of mammalian copper chaperone Cox17. Biochemical Journal, 408, 139 - 148

Voronova, A., **Kazantseva, J**., Tuuling, M., Sokolova, N., Sillard, R., L Palumaa, P. (2007) Cox17, a copper chaperone for cytochrome c oxidase: Expression, purification and formation of mixed disulfide adducts with thiol reagents. Protein Expression and Purification, 53, 138 - 144

Sarmiento, C., Nigul, L., **Kazantseva, J.**, Buschmann, M., Truve, E. (2006) AtRLI2 is an endogenous suppressor of RNA silencing. Plant Molecular Biology, 61, 153 - 163

Meloni, G., Zovo, K., **Kazantseva, J.**, Palumaa, P., Vasak, M. (2006) Organization and assembly of metal-thiolate clusters in epithelium-specific metallothionein-4. JBC, 281, 14588 - 14595

Gudima, S.O., **Kazantseva, E.G**., Kostjuk, D.A., Shchaveleva, I.L., Grishchenko, O.I., Memelova, L.V., Kochetkov S.N. (1997) Deoxynucleotidecontaining RNAs: a novel Class of templates for HIV-I reverse transcriptase. Nucleic Acids Research, 25, 4614 - 4618

Patentsed leiutised:

CANCER RELATED ISOFORMS OF COMPONENTS OF TRANSCRIPTION FACTOR COMPLEXES AS BIOMARKERS AND DRUG TARGETS; Omanik: Oncotx, Inc; Autorid: **J. Kazantseva**, A. Kazantseva; Patendinumber US 2010/0087376 A1.

MALDI MS-based high-throughput screening method for substances inhibiting aggregation of Alzheimer's A peptides; Omanik: Tallinna Tehnikaülikool; Autorid: P. Palumaa, M. Tuuling, O. Blaževitš, **J. Kazantseva**, K. Zovo, I. Šabarova; Patendinumber PCT/EE2008/000024.

Juhendatut lõputööd:

Mariann Moosar, 2011, magistritöö “Basaalse transkriptsioonifaktori kompleksi TFIIH subühiku p34 uuringud”

DISSERTATIONS DEFENDED AT
TALLINN UNIVERSITY OF TECHNOLOGY ON
NATURAL AND EXACT SCIENCES

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