Hepatocyte nuclear factor 4α multiple isoforms, their functions, and their interactomes

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Abstract

HNF4α is a master regulator gene belonging to the nuclear receptor superfamily involved in regulating a wide range of critical biological processes in different organs. Structurally, the HNF4A locus is organized with two independent promoters and is subjected to alternative splicing with the production of twelve distinct isoforms. Little is known about the mechanisms each isoform uses to regulate transcription and their biological impact, with some reports addressing these aspects. Proteomic analyses have led to identifying proteins that interact with specific HNF4α isoforms. The identification and validation of these interactions and their role in co-regulating targeted gene expression are essential to understand better the role of this transcription factor in different biological processes and pathologies. This review addresses the historical origin of HNF4α isoforms, some of the main functions of the P1 and P2 isoform subgroups and provide information on the most recent hot topic research on the nature and function of proteins associated with each of the isoforms in some biological contexts.

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Abbreviations: activator protein 1 (AP-1); AT-rich interaction domain (ARID); bromodomain containing 7 (BRD7); CREB binding protein (CBP); cytochrome P450 family 7 subfamily A member 1 (CYP7A1); DNA-binding domain (DBD); double PHD fingers 2 (DPF2); E1A binding protein P400 (EP400); enhancer of polycomb homolog 1 (EPC1); GATA zinc finger domain containing (GATAD); hepatocyte nuclear factor 4-alpha (HNF4α); histone deacetylase (HDAC); histone H4/ nucleosome acetyltransferase complex (NuA4/Tip60); human androstane receptor (hCAR); ligand binding domain (LBD); maturity-onset diabetes of the young subtype 1 (MODY1); mediator complex (MED); metastasis associated (MTA); nuclear receptor coactivator (NCOA); nuclear receptor corepressor (NCOR); nucleosome remodeling and deacetylase (NuRD); receptor interacting domain 2 (RID2); peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1); polybromo 1 (PBRM1); positive cofactor 4 (PC4); small heterodimer partner (SHP); small heterodimer partner interacting leucine zipper protein (SMILE); Switch/Sucrose-Nonfermentable (SWI/SNF); SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SMARC); TATA-binding protein (TBP); transcriptional adaptor 2 (ADA2); transcription factor 4 (TCF4); transcription factor II B (TFIIB); transthyretin (TTR).

Keywords: HNF4α, alternative spliced isoforms, proteomic, transcriptome, interactome

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Abstract

HNF4α is a master regulator gene belonging to the nuclear receptor superfamily involved in regulating a wide range of critical biological processes in different organs. Structurally, the *HNF4A* locus is organized with two independent promoters and is subjected to alternative splicing with the production of twelve distinct isoforms. Little is known about the mechanisms each isoform uses to regulate transcription and their biological impact, with some reports addressing these aspects. Proteomic analyses have led to identifying proteins that interact with specific HNF4α isoforms. The identification and validation of these interactions and their role in co-regulating targeted gene expression are essential to understand better the role of this transcription factor in different biological processes and pathologies. This review addresses the historical origin of HNF4α isoforms, some of the main functions of the P1 and P2 isoform subgroups and provide information on the most recent hot topic research on the nature and function of proteins associated with each of the isoforms in some biological contexts.

Introduction

Hepatocyte nuclear factor 4-alpha (HNF4α) is a master transcription factor part of the nuclear receptor superfamily and is primarily conserved during evolution [1, 2]. HNF4α was initially isolated and purified from the liver in the 1990s, and since its discovery, numerous studies have supported the vital role of this protein in development. One of the first pieces of evidence to this end came from its disruption in an engineered mouse knockout model in which deleted individuals did not survive beyond E9 of embryonic development [2]. It was further concluded that HNF4α was crucial for primary endoderm, liver, pancreas, and gut epithelia development [3]. After birth, HNF4α continues to be expressed in these organs, and its expression is maintained in both murine [4, 5] and human [6] adult tissues. Several independent research groups have collectively confirmed the crucial role of HNF4α in liver gluconeogenesis, glycogen synthesis, tissue architecture, epithelial morphogenesis, hepatocyte differentiation, lipid metabolism, and detoxification of xenobiotic agents [7-9]. In the pancreas, HNF4α was also shown to control insulin production by promoting gene promoter activation [10] and glucose-induced insulin secretion [11, 12]. It was also discovered that mutations in the *HNF4A*gene are responsible for maturity-onset diabetes of the young subtype 1 (MODY1) [13-15]. In the stomach, HNF4α is detected in the pit, isthmus, neck, and pepsinogen-secreting zymogenic cells of the gastric corpus. Deletion of *Hnf4a* in the mouse gastric epithelium led to increased epithelial proliferation while interfering with zymogenic cell differentiation [16]. In the large mouse intestine, loss of HNF4α during embryonic development interferes with crypt formation and goblet cell maturation and reduces epithelial cell proliferation with an impact on external muscle and vascular tissue reduction [17]. In the mouse adult intestine, HNF4α primarily acts as an activator of gene transcription. Its deletion negatively impacts polysaccharides and acid mucopolysaccharides production, mucins, and aquaporins gene expression and increases intestinal permeability [18]. Its deletion also negatively impacts expression of ion transport genes and stimulates epithelial apoptosis and mucosal immune cells infiltration [19].

All these observations support a predominant role of HNF4α during developmental organogenesis and organ function maintenance. Deregulation of HNF4α transcriptional activity is associated with several pathologies, including liver cirrhosis, hepatocellular carcinoma, MODY1, colitis, and colon cancer [20].

Alternative splicing can generate functional protein isoforms or alloforms for multiple human genes [21]. *HNΦ4A* can produce a total of 12 isoforms for which some of these were previously shown to harbor specific biological functions [22, 23]. However, the biological role of each of these isoforms in the different processes in which HNF4α is involved remains to be discovered, mainly because most of past work focused on some isoforms with the assumption that they were functionally equivalent. Large-scale identification of specific protein interactome for each HNF4α isoform remains crucial to distinguish the specific nature of targeted genes and their associated biological functions according to the physio-pathological state dependent on HNF4α [22, 23].This review will focus on the work done so far to identify interactions of specific HNF4α

isoforms with protein components involved in transcriptional regulation and their impact on specific gene expression and biological functions.

2. HNΦ4α isoφorμs αre προδυcεδ φrοm 2 ινδεπενδεντ Π1 ανδ Π2 προμοτερς

HNF4α was originally isolated and purified from rat liver nuclear extracts and characterized as a factor that binds to specific promoter elements of the transthyretin (*TTR*) and apolipoprotein C2 genes [24]. Like other members of the nuclear receptor family, HNF4α was originally described as containing an A/B domain including an AF-1 transactivation region, which allows interaction with different proteins involved in regulating transcription; a C domain or DNA-binding domain (DBD) containing specific recognition elements that bind to promoter regions of their target genes; a D domain that acts as a hinge between the C and E domains; an E domain capable of interacting with a ligand binding domain (LBD) and that contains an AF-2 ligand-dependent activation region; and an F domain with repressive functions of protein activity [25, 26] (Figure 1A). *HNF4A* gene is located on chromosome 20 [27], more precisely on the long arm between regions 12 and 13 [28]. A critical feature of *HNΦ4A* is the presence of P1 and P2, two independent promoters that can drive gene transcription and are separated by more than 45 kb [29]. By alternative splicing, these promoters can give rise to a total of 12 isoforms which, accordingly to the promoter driving their expression, have been classified into two subgroups of isoforms called P1 (α1 to α6) and P2 (α7 to α12) [30, 31] (Figure 1B). P1 (α1 to α3) and P2 (α7 to α9) isoforms have been extensively studied and characterized in various organs and tissues. They are referred to as the canonical isoforms, as opposed to the non-canonical P1 (α4 to α6) and P2 (α10 to α12) isoforms for which little is known about their distribution and functions [31].

2.1 HNΦ4α Π1 ανδ Π2 συβγρουπς οφ ισοφορμς ανδ τηειρ οριγινς

Since their discovery, HNF4α isoforms have been referred by various nomenclatures, mainly depending on the organism from which they were isolated. This historical confusion contributed to a cumulative lack of uniformity in their description from the literature as well as from main repository databases, leading in some cases to significant discrepancies between the matching of specific gene transcript sequences of some of these isoforms [31]. To simplify and resolve confusion around the classification of every single HNF4α isoforms, a recent nomenclature was proposed to organize these isoforms accordingly to the specificity of their N-terminal and C-terminal regions [31, 32]. From partial sequencing of the *HNF4A* locus, six putative isoforms were originally predicted being produced from alternative splicing [33]. This included the 455 amino acids rat protein, equivalent to the human 464 amino acids called HNF-4B, which was isolated and purified from rat liver nuclear extracts [24], and later renamed HNF4α1 (P1a-α1, Figure 1B). In addition, HNF4α2 (P1a-α2, Figure 1B) was initially described in rat liver as an isoform containing an insertion of 10 amino acids in the C-terminal region of the protein [34, 35]. This protein was after that described as HNF-4CL4, a 474 amino acid protein isolated from the human liver [36]. HNF4α3 (P1a-α3, Figure 1B) was first described in the human liver as an isoform containing a sequence insertion of 40 amino acids starting at position 369 and initially called HNF4C [27]. HNF4α4 (P1b-α4, Figure 1B) included an additional sequence of 30 amino acids in its N-terminal region as opposed to P1a-α1 to α3 isoforms [27]. More recently, an error was observed from the initial reported sequence of P1b-α4 that predicted a premature stop codon and a truncated protein. The correct sequence would have to contain an alternative starting codon leading to the production of a protein with a different sequence of the N-terminal region from what was initially described [31]. HNF4α5 (P1b-α5, Figure 1B) and HNF4α6 (P1b-α6, Figure 1B) isoforms were deducted from the alternative splicing mechanisms knowledge obtained from the first isolated isoforms. As initially reported [33], these isoforms contain exons 1B and 1C in their N-terminal region, while P1b-α5 contains the same insertion described for P1a-α2, and P1b-α6, the same insertion described for P1a-α3 isoform [33]. Subsequently, a gene transcript containing a 154 bp sequence variant in the N-terminal region and different from all HNF4α isoforms described at that time, was isolated from immortalized murine liver cells and named HNF4α7 (P2a-α7, Figure 1B) [37]. From extrapolation of an additional combination of possible splicing events, two additional isoforms were described: HNF4α8 (P2a-α8, Figure 1B) and HNF4α9 (P2a-α9, Figure 1B), both containing an N-terminal region identical to HNF4α7 (exon 1D, Figure 1B). The C-terminal regions of these two isoforms differed, with HNF4α8 containing an identical C-terminal region to HNF4α2 isoform and HNF4α9 C-terminal region being

identical to HNF4α3 isoform [38]. With the finding of an additional P2 promoter located in the *HNF4A* locus [29], most subsequent studies started distinguishing between P1 (α1 to α6) and P2 (α7 to α9) isoforms. Three additional P2-driven isoforms (P2b-α10, P2b-α11, and P2b-α12) were reported to include both exons 1D and 1E in their N-terminal region (Figure 1B) [30]. The variable regions among these isoforms were localized again in the C-terminal region, where HNF4α10 isoform contained the common C-terminal region of HNF4α1 and α7, HNF4α11 with the common C-terminal region of HNF4α2 and α8, and HNF4α12, the same C-terminal region of HNF4α3 and α9 (Figure 1B).

2.2 Σπεσιφις δισ τριβυτιον οφ ΗΝΦ4α ισοφορμς ιν οργανς ανδ τισσυες

As describe above, the structural differences among HNF4α isoforms have raised the hypothesis that distinct functions related to these differences might occur in the context of co-interacting partners in transcriptional regulation during embryonic development and adult organ tissue maintenance [22]. Numerous studies have evaluated the differential expression of HNF4α isoforms in a variety of tissues, with the general observation that expression of HNF4α is predominant in the liver, small intestine, colon, and kidneys [6, 27, 30, 34, 35, 37, 39] and to a lesser extent, in the stomach, pancreas, testes, bladder, prostate, ovaries, heart, spleen, lungs, skin, and skeletal muscles [31, 32, 39]. The initial development of specific antibodies for P1 and P2 classes of isoforms has allowed clarifying this profile of expression at the protein level. In the liver, it was observed that P2 isoforms (α7 to α9) are co-expressed with P1 isoforms (α1 to α6) during embryonical stages, while only P1 isoforms become detectable in the adult phase [40]. In healthy renal tissues, P1 isoforms expression was also found to be much higher than P2 isoforms expression, while adult pancreatic and gastric tissues showed a much higher level of expression of P2 isoforms than P1 isoforms [39]. While the intestine displays a higher expression of P2 isoforms at the embryonical stage, it appears that this is the only organ at the adult stage able to express comparable levels of both P1 and P2 isoforms [39]. However, the lack of specific antibodies able to discriminate between the specific isoforms that compose both HNF4α P1 and P2 family members in these physiological contexts still prevent the scientific community from further pinpointing these expression profiles.

For this reason, many groups have turned to the use of techniques allowing the measurement of specific gene transcripts for some of these specific isoforms. For example, HNF4α1 (P1a-α1) was found to be much more abundant than both HNF4α2 and HNF4α3 (P1a-α3) isoforms in both the liver and kidneys [27, 36]. Although the existence of P1b-α4 to P1b-α6 isoforms is still controversial based on expression studies, a recent report has suggested these isoforms harbor potent repression activity for the regulation of liver-specific genes and, at the same time, participate in the activation of genes involved in the pro-inflammatory response [32]. P2a-α7 and P2a-α8 isoforms in adult pancreatic tissues are much more expressed than P2a-α9 and P2b-α10 to P2b-α12. More particularly, P2a-α9 isoform is less expressed in the islets of Langerhans than in the rest of the pancreas, whereas P1a-α1 to P1a-α3 isoforms are only detected in the embryonic tissue. However, both P1a-α1 and P1a-α2 isoforms have been reported to be expressed in adult mouse islets of Langerhans [30, 41-44]. In the digestive tract, expression of the HNF4α isoforms vary according to the region studied. In the stomach, higher expression of both P1a-α1[44] and P2a-α7 [45] isoforms was reported. In contrast, expression of P1a-α2, P2a-α7, P2a-α8, and P2a-α9 was more critical from the duodenum to the rectum, with some expression of P1a-α1 in the regional portions of the colon [31]. P1b-α4, P1b-α5, P1b-α6, and P2b-α10 [32] were also reported expressed in the small intestine and colon, while P2b-α12 isoform was strictly detected in the small intestine [32]. It is noteworthy to mention that no expression for any HNF4α isoforms has never been detected in the esophagus [31].

3. Σπεσιφις βιολογισαλ φυνστιονς οφ ΗΝΦ4α Π1- ανδ Π2-δεριεδ ισοφορμς

Given the complex nature of HNF4α isoforms regulation of expression and tissue distribution, a few studies have investigated the specific roles of these isoforms, and with often the limitation of targeting multiple members contained in either P1 or P2 isoform subgroups. Nevertheless, important observations were obtained from these studies. Using an exon swapping strategy, Briancon *et al* . were able to generate knock-in mouse models switching the AF-1 activation domain between HNF4α1 (1a-α1) and HNF4α7 (2a-α7) isoforms driven by P1 or P2 promoters, respectively [45]. From this strategy, it was found that mice expressing only the P1

isoform resulted in impaired glucose tolerance. In contrast, those expressing only the P2 isoform displayed a significant decrease in serum lipids and steatosis and showed alterations in the transcription of genes involved in lipid metabolism and transport [45]. When the same mouse models were used in the context of gut biology, no basal morphological changes were reported during normal homeostasis of adult mice [46]. This was not unexpected, given that the conditional knockout of *Hnf4a* in intestinal epithelium did not severely impact this tissue [47]. However, when experimental colitis was inflicted in these mice, an increase in colitis severity and mortality was observed in mice expressing P2-only isoform [46]. In the same report, when cancer-associated colitis was induced among these mouse models, P1-only isoform individuals exhibited a reduction in intestinal tumor size and number compared to controls. On the contrary, P2-only isoform individuals showed a larger and increased number of intestinal tumors when compared to controls [46]. Although these two previous studies highlighted some specific biological roles for P1-derived 1a-α1 and P2-derived 2a-α7 isoforms, much needs to be done to picture the global complexity of whole HNF4α isoforms. In a complementary set of experiments, Babeu *et al* . investigated the specific roles of P1 versus P2 groups of isoforms in the human context [48]. P1 subgroup of isoforms was found to be expressed in association with differentiated colonocytes, while P2-associated isoforms were preferentially detected in proliferating colonocytes. This pattern of expression in association with proliferation versus differentiation cell status was maintained during colon cancer, with P1 isoforms being repressed and P2 isoforms induced in the neoplastic tissues compared to their normal margins [48]. Under these circumstances, the mechanism involved in the repression of P1 isoforms was found to be dependent on β-catenin expression [48]. Using a small interfering RNA strategy, the same report identified specific classes of genes dependent on whole P1 versus P2 isoforms transcriptional activity [48] . P1-derived isoforms were involved in regulating genes associated with cell metabolism whereas P2-derived isoforms were specifically involved in regulating genes related to cell-cycle progression and survival [48]. Again, although this study highlighted specific global roles for P1 versus P2 whole classes of isoforms, further studies are needed to understand the specific role of every single isoform in these biological contexts.

4. HNΦ4α ισοφορμς ανδ τηειρ αστιον ον γενε εξπρεσσιον

Most of the HNF4α isoforms play an essential role in transcriptional regulation, mainly through activation and, to a lesser extent, repression of many genes involved in various biological processes. For example, P1a-α1 was found to enhance the transcription of the human androstane receptor (hCAR) gene promoter in human liver cells, whereas P2a-α7 acted as a repressor in this context [49]. One additional pioneer study specifically addressed the potential differential impact of two different P1 and P2 HNF4α isoforms at a larger scale by measuring the transcriptomic impact of forced expression of either P1a-α2 or P2a-α8 in a human intestinal epithelial cell line originally devoid of HNF4α expression [50]. It was concluded that P1a-α2 influenced a set of genes linked to the inhibition of cell growth and death. In contrast, P2a-α8 specifically influenced genes involved in the progression of the cell cycle and development [50]. In a following study, Lambert*et al* . investigated the impact of every single HNF4α isoform on global transcriptomic changes in the same human intestinal epithelial cell line [31]. This analysis indicated that both P1a-α1 and P1a-α2 were the most potent isoforms to influence the transcriptome, while P2 isoforms were less efficient overall. Interestingly, the non-canonical P1 isoforms (P1b-α4, P1b-α5, and P1b-α6) did not significantly impact gene expression, possibly because of their incapacity to physically interact with the classical HNF4α element [31]. P2a-α9 was found to act mainly as a repressor of gene expression, while both P2b-α10 and P1-α3 mainly exerted a positive action on gene expression. These observations collectively suggested that the presence of the A/B domain, rather than the repressive F domain, was the main criterion to explain these differences. HNF4α isoforms with a shorter F domain were primarily ineffective in influencing gene expression, suggesting that this domain can play a crucial role during transcriptional regulation [31]. An additional study investigated the combinatory impact of these isoforms on the expression of a subset of genes involved in inflammation and immune response [32]. Again, a specific regulatory pattern of gene expression was observed depending on the nature of these isoforms in this context. For example, the combination of P1a-α2 and P1a-α3 or P2a-α9 and P2b-α12 as heterodimers was more efficient in repressing specific target genes than their homodimer counterparts. On the other side, P1a-α3 and P2a-α8 or P1b-α6 and P2b-α12

heterodimers were found more potent in transcriptional activation again when compared to their homodimers equivalent. One concrete example is the cytochrome P450 family 7 subfamily A member 1 (CYP7A1), an enzyme involved in cholesterol metabolism, that was found specifically upregulated with P1a-α3 and P2a-α8 heterodimers [32]. Still, several mechanistical aspects relative to the specificity of action of these various isoforms, including the specific nature of assembled proteins in the composition of HNF4α transcriptional complexes for a given gene, remain to be further explored.

5. HNΦ4α ισοφορμς ανδ τηειρ ιντεραστινγ προτεινς

As for other nuclear receptors, HNF4α is recruited on binding response elements of its target genes. It interacts via its AF-1 and AF-2 domains with coactivating and corepressor protein complexes to regulate transcription (Figure 2). It is well accepted that the presence of AF-1 and or AF-2 domains in HNF4α isoforms can widely impact the physical interaction between these isoforms and other proteins [42, 51, 52]. This could mechanistically explain how these specific isoforms can differentially regulate gene expression in various tissues and developmental stages [53, 54]. However, few studies have deeply characterized these interactions at the molecular and biological levels. Therefore, the following sections highlight some of these interactions in the context of specific HNF4α isoforms.

5.1 HNΦ4α1(Π1α-α1) προτειν παρτνερς

P1a-α1 represents the isoform for which most studies have been carried out to identify transcriptional coregulators. This is probably because this isoform was the first to be characterized to contain both AF-1 and AF-2 transactivation domains, making it a strong candidate to extrapolate for similar interactions in the context of the other isoforms and their capacity to recruit the polymerase II. For example, It was observed that during initial steps of transcription preinitiation complex assembly, P1a-α1 binds to Transcription factor II B (TFIIB) and allow its interaction with TATA-binding protein (TBP) with the help of both AF-1 and AF-2 transactivation domains (Figure 2) [55]. On the other hand, both AF-1 and AF-2 can interact with the CREB binding protein (CBP), which in turn acetylates P1a-α1 isoform to prevent its export to the cytoplasm and increase its affinity and stabilize its interaction with DNA [56]. While the interaction between CBP and the AF-1 domain reduces the overall potential in its transcriptional activation [57], AF-1 presence is indispensable for bridging these two proteins and confers a greater affinity for the transcriptional complex as opposed to other isoforms such as P2a-α7, which is devoid of this domain [26, 58]. It was also reported that the Nuclear Receptor Coactivator (NCOA) 1 directly associates with the AF-2 domain of P1a-α1 both *in vivo* [11] and *in vitro* [26] (Figure 2). Their co-expression led to the activation of HNF1α in the HepG2 hepatoma cell line associated with the regulation of glucose homeostasis [11]. To this end, it was found that the transcriptional activity of P1a-α1 was increased by its combination with NCOA1, NCOA2, p160, and p300 [11, 58]. Although synergistic studies involving NCOA3 and the above-mentioned proteins including P1a-α1 has not been thoroughly investigated, NCOA3 was shown interacting with P1a-α1 [59, 60]. P1a-α1 also interacts via its AF-2 domain with Receptor interacting domain 2 (RID2) of the Nuclear Receptor Corepressor (NCOR) 2. This interaction abolishes transcriptional activation mediated by the cofactors CBP, NCOA2 and p300 by competing for the same host region of these coactivators [61]. Furthermore, it has been observed that the presence of the AF-1 domain potentiates the repression effect of NCOR2 (Figure 2); this could explain why the P1a-α1 isoform is recruited when there is an interaction between NCOR2 and histone deacetylase (HDAC) 3 [58]. Similar findings were reported for corepressors such as small heterodimer partner (SHP) [60] and small heterodimer partner interacting leucine zipper protein (SMILE) [62]. However, the nature of the mechanisms that trigger these interactions are not entirely clear.

5.2 HNΦ4α2 (Π1α-α2) προτειν παρτνερς

Some studies have shown interactions between the P1a-α2 AF-1 domain and proteins involved in regulating transcription, including TBP, TFIIB, TAFII3, TAFII31, TAFII80, TFIIH-p62, CBP, transcriptional adaptor 2 (ADA2) and positive cofactor 4 (PC4) (Figure 2). These interactions are mediated by hydrophobic and aromatic residues localized between amino acids 1-12 (Tyr6) and 13-24 (Tyr14, Lys10, Lys17, and Phe19) of the P1a-α2 AF-1 domain [52]. Although these interactions could occur with other HNF4α isoforms that

contain the AF-1 domain, it is unclear whether there are structural differences in other isoforms that could influence these interactions. For example, it was observed that P1a-α2 AF-1 interaction with the C/H1 domain of CBP generates significantly higher increase in transcriptional activity when compared to the P1a-α1 isoform, due to a greater affinity of P1a-α2 for CBP [63]. This affinity could be related to the insertion of 10 amino acids in the F domain of P1a-α2 (Figure 1B), which would generate a conformational change in the protein to prevent the blockade this domain typically exerts for the interaction with coactivators. Similar observations were made for the interaction between P1a-α2 and NCOA2, for which transcriptional activation was reported to be seven times stronger for P1a-α2 compared to P1a-α1 [26]. It was also suggested that P1a-α2 interacts with activator protein 1 (AP-1), with the biological consequence of reducing the growth of intestinal tumor cells [50]. Although ChIP-seq analyses suggested an interaction between these two proteins, a direct physical interaction was not confirmed in this context. Other studies have proposed the formation of a direct interacting protein complex between peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and P1a-α2, this interaction impacting gluconeogenesis and glucose homeostasis in the liver [64].

5.3 HNΦ4α7 (Π2α-α7) προτειν παρτνερς

A certain transcriptional synergy was reported between P2a-α7 and the coactivators p300 and NCOA2, although the magnitude of transcriptional activation was lesser when compared to P1a-α1 [58]. It was also shown in the same report that NCOR2 was able to repress gene transcription via interaction with P2a-α7 and the recruitment of HDAC4; however, this repression activity was reduced when compared to P1a-α1.

5.4 HNΦ4α8 (Π2α-α8) προτειν παρτνερς

As for other HNF4α isoforms, little is known about the nature of protein interactions between P2a-α8 isoform and co-transcriptional regulators. One study showed that P2a-α8 could recruit transcription factor 4 (TCF4) directly on HNF4α DNA-binding elements, although no direct physical interaction between these proteins could be demonstrated [50].

6. HNΦ4α ισοφορμς ανδ τηειρ σπεςιφις ιντεραςτομε

Despite the above findings, few studies have assessed the landscape of HNF4α isoforms' specific interactome and their impact on gene expression. Only three studies have addressed these aspects [31, 32, 65]. The pioneering study performed by Daigo *et al.* used two independent HNF4α antibodies, one raised against the F domain and another against the A/B domain, to pulldown native endogenous HNF4α isoforms from the human hepatic HepG2 cell line. [65]. Semi-quantitative proteomic analysis revealed that HNF4α could interact with a wide variety of protein complexes, some associated with the DNA-dependent protein kinase catalytic subunit, histone acetyltransferase complexes, alternative splicing, chromatin remodeling, and nucleosome remodeling. These authors also identified the formation of heterodimers between HNF4α and HNF4γ and further explored the impact of this novel interaction on the transcriptional regulation of genes involved in liver metabolism [65]. With the use of large-scale approaches, two additional studies indicated that each of these HNF4α isoforms could influence a variety of genes with different patterns of expression and that for most tested isoforms, a positive modulatory effect was observed on their target genes with some exceptions. For example, the study from Ko *et al* . [32] reported that non-canonical isoforms of the P1b group negatively modulate gene expression. In contrast, Lambert *et al.* [31] showed that only the P2a-α9 isoform acted likewise. These discrepancies may result from the nature of these analyses, where Lambert *et al* . investigated the entire transcriptome in their cellular system. In contrast, Ko*et al.* focused on a cluster of genes restricted to inflammation and immune response. Nevertheless, the study from Lambert *et al.*raised the possibility that the P1b subgroup of isoforms may act as negative regulators of the expression of several genes through interaction with other transcription factors [31]. Another significant difference between these two studies is the cellular system. The different or contradictory results reported could be related to the biological models and the overall design used in each study.

Lambert *et al.* chose to stably single-insert a doxycycline-inducible copy of each HNF4α isoform into an intestinal epithelial cell line (HCT116) devoid of endogenous HNF4α expression. This approach presented

the advantage of comparing the transcriptome in the absence and presence of any given isoform in an endogenous range level that would be expected under this biological context. On the other hand, Ko *et al* . used a hepatocyte cell line (HuH7) that endogenously expressed HNF4α and transiently transfected each of the isoforms in different combinatory setups. This approach presents the disadvantage of producing possible supraphysiological levels of each of these isoforms and possible interference of endogenous HNF4α isoforms naturally produced in this cell line. However, both studies recognize a complex variability of isoforms in the N- and C-terminal regions and agree that this could be why the non-canonical isoforms of the P1b-α4 to α6 subgroup harbor different affinity with the DR1 motif, the classical and functional response element for HNF4α. Additionally, Ko *et al.* noted that canonical P1b group isoforms could bind and activate transcription, even when the binding sites in their DR1 motif 1 and 2 are mutated [32].

The study by Lambert *et al.* used powerful and complementary quantitative proteomic approaches to precisely identify the interactome of each HNF4α isoform in the context of HCT116 cells [31]. The authors observed that HNF4α isoforms interacted with a joint group of 69 proteins, for which some were previously identified and discussed above. For example, interactions with transcriptional activating complexes CBP/p300 and repressing complexes NCOR1 and 2 were observed (Figure 2). Other and newly identified large complexes were identified to interact with most individual HNF4α isoforms [31]. For example, the Switch/Sucrose-Nonfermentable (SWI/SNF) family of chromatin remodeling complexes, including the subunits AT-rich interaction domain (ARID)1A, ARID1B, ARID2, bromodomain containing 7 (BRD7), double PHD fingers 2 (DPF2), polybromo 1 (PBRM1), SWI/SNF related, matrix associated, actin dependent regulator of chromatin (SMARC)A4, SMARCC1, SMARCC2, SMARCD2, and SMARCE1 were all identified in this context (Figure 2). These complexes can activate or inhibit transcription in the presence of activators or repressors, and alterations in some of their units have been associated with different types of cancer and neurological disorders [66-68]. In addition, interactions between several HNF4α isoforms and subunits of the mediator complex, including mediator complex (MED)1, MED14, and MED15, were identified (Figure 2) [31]. Interestingly, Daigo *et al.* previously identified MED16 and MED24 as interactors of HNF4α [65], and some of these interactions were also observed to take place using GST-pulldown and co-immunoprecipitations assays [69, 70]. Given this complex's importance in regulating the expression of many genes, mutations in some of these subunits are often associated with different diseases, including cancer [71, 72]. Interactions between HNF4α isoforms and enhancer of polycomb homolog 1 (EPC1), BRD8, and E1A binding protein P400 (EP400), all subunits of the histone H4/ nucleosome acetyltransferase complex (NuA4/Tip60), were also observed [31]. This complex involves transcriptional regulation, chromatin modification, cell migration and invasion, mitosis, and genomic instability (10). In addition, some of these subunits are also associated with colorectal cancer progression [73].

Finally, additional interactions involving some of the HNF4α P1 and or P2 isoforms with members of the nucleosome remodeling and deacetylase (NuRD) complex, including metastasis associated (MTA)1, MTA2, GATA zinc finger domain containing (GATAD)2A, and GATAD2B have also been observed (Figure 2) [31]. Likewise to SWI/SNF complexes, NuRD is involved in regulating transcriptional events, genome integrity, and cell cycle progression. Defects in the activity of the complex have been linked to defects in embryonic development, premature aging, oncogenesis, and cancer progression [74]. Given the importance of the complex in tumor diseases, some of the members of this complex, such as MTA1, have been proposed as potential therapeutic targets [75].

7. Conclusions and future perspectives

Since its initial discovery more than three decades ago, HNF4α has been extensively studied in the context of regulating gene transcription, metabolism, and tissue differentiation. Subsequent structural analyses of the *HNF4A* locus revealed a high level of complexity, with the significant finding that HNF4α no longer refers to one single protein but englobes different classes of isoforms produced mainly by the usage of two different promoters and alternative splicing. As highlighted above, recent advances have supported specific roles attributed to each HNF4α P1 and P2 subgroup, at least in gut epithelial tissues where several isoforms from both subgroups are detected. Additionally, some reports have identified coactivators and corepressors

interacting with HNF4α isoforms. Some of these studies considered the specificity of each single isoforms in their experimental design, with the conclusion that some of these interactions depend on the specific nature of these isoforms. Although there is a broad agreement on the importance of several identified HNF4α copartners and their function in regulating gene transcription, the specific nature of these interactions among various isoforms and their biological impact awaits to be addressed. Even though recent advances highlighted the interactome nature of these HNF4α isoforms, more studies are needed to further dissect at both mechanistic and biological levels the differences between all these isoforms. The assessment of whether these interactions occur directly between identified proteins and their specific HNF4α isoforms is critical to inform their global biological role further.

On the other hand, there is common knowledge on the specificity of gene promoter usage in the production and localization of HNF4α P1 and P2 isoforms subclasses among various tissues. However, the exact distribution of every HNF4α isoform of these subclasses during pathophysiological processes is not well established. One of the main reasons to explain this lack of knowledge comes from the difficulty of raising specific antibodies for every single isoform since none harbor their own unique protein domains. Given the central role of HNF4α in the maintenance of several tissues and diseases, one of the future challenges will be to distinguish the contribution of each of the HNF4α isoforms to this end. These strategies will become particularly attractive for the development of therapeutical targeting interventions.

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Figure Legends

Figure 1 . The human *HNF4A* locus harbors two promoters that produce twelve isoforms by alternative splicing.A . Schematic representation of the *HNF4A* gene. The nomenclature historically assigned to each exon and the options for alternative splicing between P1 and P2 promoter regions are shown.**B** . Human isoforms produced by *HNF4A* . The critical structural differences in N-terminal and C-terminal regions between the different isoforms' subgroups are discriminated by different colors. The total number of amino acids are from the human isoforms as described in the literature, and the matching number for the murine equivalents are indicated in parentheses. The nomenclature used for each isoform corresponds with the one Lambert *et al.* proposed [31].

Figure 2 . Ιδεντιφιεδ προτεινς ιντεραστινγ ωιτη HNΦ4α ισοφορμς ανδ ινολεδ ιν αχτιατιον ορ ρεπρεσσιον οφ τρανσχριπτιον . The structure of protein complexes, as displayed is based on recent studies on their crystallography. Only proteins with possible interactions with HNF4α are indicated. A color code distinguishes specific characteristics associated with HNF4α isoforms. In purple, the physical interaction with all isoforms was not demonstrated. In green, the physical interaction with P1a-α1 to α3, P1b-α4 and α5, P2a and P2b isoforms was demonstrated. In yellow, the physical interaction with P1a-α1 and α3, P1b-α4 to α6, P2a and P2b isoforms was demonstrated. In blue, physical interaction with P2a-α7 to α9 was not demonstrated. In orange, physical interaction with P1a-α1 to α3, and P2a and P2b isoforms was not demonstrated. In light blue, physical interaction with P1b, P2a and P2b isoforms was not demonstrated. In pink, physical interaction with P2b α10 to α12 was not demonstrated. In light green, physical interaction with P1a-α2, P1b-α5, P2a-α8, and P2b-α12 was not demonstrated. In dark red, physical interaction with all HNF4α isoforms was demonstrated.

Conflict of interest

The authors declare no conflict of interest.

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