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"Genetic alterations in Type 2 Diabetes: regulation of *PED/PEA-15* gene expression"

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CHAPTER 1

Background and Aim of the Work

Diabetes Mellitus (DM) comprises a group of common metabolic disorders that share the phenotype of hyperglycaemia. Several distinct types of DM exist and are caused by a complex interaction of genetic and environmental factors, and life-style choices. Depending on the etiology of the DM, factors contributing to hyperglycaemia may include reduced insulin secretion, decreased glucose utilization and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system. In the United States, DM is the leading cause of end-stage renal disease (ESRD), nontraumatic lower extremity amputations, and adult blindness. With an increasing incidence worldwide, DM will be a leading cause of morbidity and mortality for the foreseeable future [1].

DM is classified on the basis of the pathogenic process that leads to hyperglycaemia. The two broad categories of DM are designed type 1 and type 2. Type 1A DM results from autoimmune beta cell destruction, which leads to insulin deficiency. Individuals with type 1B DM lack immunologic markers indicative of an autoimmune destructive process of the beta cells. However, they develop insulin deficiency and are ketosis prone. Relatively few patients with type 1 DM are in the type 1B idiopathic category. Type 2 DM is an heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production. Distinct genetic and metabolic defects in insulin action and/or secretion give rise to the common phenotype of hyperglycaemia in type 2 DM [1].

Type 1 Diabetes Mellitus

Type 1A DM develops as a result of the synergistic effects of genetic, environmental, and immunologic factors that ultimately destroy the pancreatic beta cells. The temporal development of type 1A DM is shown schematically as a function of beta cell mass. Individuals with a genetic susceptibility have normal beta cell mass at birth but begin to lose beta cells secondary to autoimmune destruction that occurs over months to years. This autoimmune process is thought to be triggered by an infectious or environmental stimulus and to be sustained by a beta cell-specific molecule. In the majority of individuals, immunologic markers appear after the triggering event but before diabetes becomes clinically overt. Beta cell mass then begins to decline, and insulin secretion becomes progressively impaired, although normal glucose tolerance is maintained. The rate of decline in beta cell mass widely varies among individuals, with some patients progressing rapidly to clinical diabetes and others evolving more slowly. Features of diabetes do not become evident until a majority of beta cells are destroyed (80%) and the individual becomes completely insulin deficient [1].

Genetic susceptibility to type 1A DM involves multiple genes. The concordance of type 1A DM in identical twins ranges between 30 and 70%, indicating that additional modifying factors must be involved in determining whether diabetes develops. The major susceptibility gene for type 1A DM is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40 to 50% of the genetic risk of developing type 1A DM. Most individuals with type 1A DM have the HLA DR3 and/or DR4 haplotype. Refinements in genotyping of HLA loci have shown that the haplotypes DQA1*0301, DQB1*0302, DQA1*0501, and DQB1*0201 are most strongly associated with type 1A DM. The risk of developing type 1A DM is increasing tenfold in relatives

of individuals with the disease. Nevertheless, most individuals with predisposing haplotypes do not develop diabetes. In addition, most individuals with type 1A DM do not have a first-degree relative with the disorder [1].

Type 2 Diabetes Mellitus

Type 2 DM is characterized by three pathophysiologic abnormalities: impaired insulin secretion, peripheral insulin resistance, and excessive hepatic glucose production. Obesity, particularly visceral or central (as evidenced by the hip-waist ratio), is very common in type 2 DM. In the early stages of the disorder, glucose tolerance remains normal, instead of insulin resistance, because the pancreatic beta cells compensate by increasing insulin output. As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state. Later on, impaired glucose tolerance, characterized by elevations in postprandial glucose, develops. A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycaemia. Ultimately, beta cell failure may ensue [1].

Metabolic abnormalities

Insulin resistance – The decreased ability of insulin to effectively act on peripheral target tissues (especially muscle and liver) is a prominent feature of type 2 DM and results from a combination of genetic susceptibility and obesity. Insulin resistance is relative, however, since the supernormal levels of circulating insulin will normalize the plasma glucose. Insulin dose-response curves exhibit a rightward shift, indicating reduced sensitivity, and a reduced maximal response, indicating an overall decrease in maximum glucose utilization (30 to 60% lower than

healthy individuals). Insulin resistance impairs glucose utilization by insulin sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycaemia. Increased hepatic glucose output predominantly accounts for increased fasting plasma glucose levels, whereas decreased peripheral glucose utilization results in postprandial hyperglycaemia. The precise molecular mechanism of insulin resistance in type 2 DM has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia rather than a primary defect. Therefore, postreceptorial defects are believed to play the predominant role in insulin resistance.

Another emerging theory proposes that elevated levels of free fatty acids (FFA), a common feature of obesity, may contribute to the pathogenesis of type 2 DM. FFA can impair glucose utilization in skeletal muscle, promote glucose production by the liver, and impair beta cell function [1].

Impaired insulin secretion – Insulin secretion and sensitivity are interrelated. In type 2 DM, insulin secretion initially increases in response to insulin resistance to maintain normal glucose tolerance. At the beginning, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion. The response to other non-glucose secretagogues, such as arginine, is preserved. Eventually, the insulin secretory defect progresses to a state of grossly inadequate insulin secretion. The reason(s) for the decline in insulin secretory capacity in type 2 DM is unclear. The metabolic environment of diabetes may also negatively impact islet function. For example, chronic hyperglycaemia paradoxically impairs islet function (“glucose toxicity”) and leads to a worsening of hyperglycaemia. Improvement in glycaemic control is often

associated with improved islet function. In addition, elevation of FFA levels ("lipotoxicity") and dietary fat may also worsen islet function.

Increased hepatic glucose production – In type 2 DM, insulin resistance in the liver, reflects the failure of hyperinsulinemia to suppress gluconeogenesis, which results in fasting hyperglycaemia and decreased glycogen storage by the liver in the postprandial state. Increased hepatic glucose production occurs early in the course of diabetes, though likely after the onset of insulin secretory abnormalities and insulin resistance in skeletal muscle.

Genetic considerations

As mentioned above, insulin resistance and impaired beta cell function are the prominent features of type 2 DM, and they are contributed by both genetic and environmental factors. These factors might affect either the process of insulin signal transmission across the plasma membrane and/or the biochemical pathways allowing glucose uptake and metabolism by the cells, or might affect the pathways regulating beta cell function, including those for beta cell compensation. While several environmental factors have been identified, discovery and characterization of the genes involved in type 2 DM has been an arduous task and has proceeded slowly. In the past ten years, indeed, geneticists have devoted a large amount of efforts to find type 2 DM genes. These efforts have included many candidate-gene studies, extensive efforts to fine map linkage signals, and an international linkage consortium that was perhaps the best example of a multi-centre collaboration in common-disease genetics (Genome Wide Association Studies – GWAS). Among these efforts, only the candidate-gene studies produced unequivocal evidence for common variants involved in type 2 DM. These are the E23K variant in the potassium inwardly-rectifying channel, subfamily J, member 11

(KCNJ11) gene [2], the P12A variant in the peroxisome proliferator-activated receptor γ (PPARG) gene [3], and common variations in the hepatic transcription factor 2 (TCF2) [4] and the Wolfram syndrome 1 (WSF1) 10 genes [5]. All of these genes encodes proteins that have strong biological links to diabetes. Rare, severe mutations in these four genes cause monogenic forms of diabetes, and two of them are targets of currently used anti-diabetic drugs: KCNJ11 encodes a component of a potassium channel with a key role in beta cell physiology and is targeted by the sulphonylurea class of drugs; PPARG encodes a nuclear receptor involved in adipocyte differentiation and is targeted by the thiazolidinedione class of drugs (Fig.1).

A common amino-acid polymorphism (Pro12Ala) in peroxisome proliferator-activated receptor g (PPAR-g) has been associated with type 2 DM. People homozygous for the Pro12 allele are more insulin resistant than those having one Ala12 allele and have a 1.25-fold increased risk of developing diabetes. Moreover, there is also evidence for interaction between this polymorphism and fatty acids, thereby linking this locus with diet [3].

In 2006, by far the most spectacular recent development in the genetics of type 2 DM has been the identification of TCF7L2 (encoding transcription factor-7 like 2) as the most important type 2 DM susceptibility gene to date [6]. The estimate of effect size (an odds ratio for type 2 DM of 1.4-fold per allele) was identified in an intronic SNP with uncertain functional credentials (rs7903146). TCF7L2 variation is strongly associated with rates of progression from impaired glucose tolerance to diabetes (with a hazard ratio of 1.55 between homozygote groups). TCF7L2 is widely expressed and involved in the Wnt signalling cascade. Most studies suggest that the predominant intermediate phenotype associated with TCF7L2 variation is impaired insulin secretion,

consistent with the replicated observation that the TCF7L2 association is greater among lean than obese type 2 DM subjects.

TCF7L2 result was encouraging for two reasons. Firstly, the study analyzed more than 200 markers across a region of linkage on chromosome 10q, but the variants that were found to alter risk did not explain the linkage signal, suggesting that a non-candidate gene or region-based association effort (such as GWAS) could work. Secondly, TCF7L2 was a completely unexpected gene, showing that a genome-wide approach could uncover previously unexpected disease pathways.

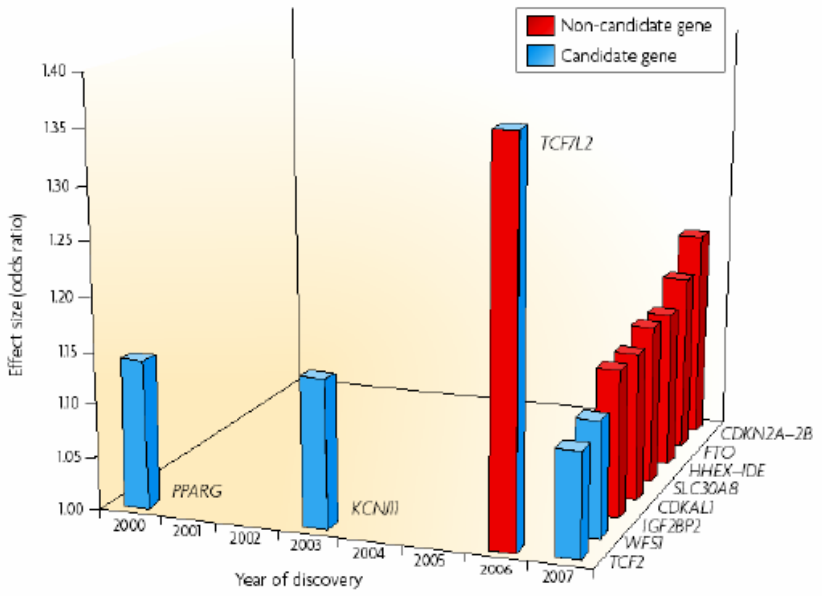

Figure 1 – Effect sizes of the 11 common variants confirmed to be involved in type 2 diabetes risk.

The x axis shows the year when published evidences reached the levels of statistical confidence that are now accepted as necessary for genetic association studies. CDKAL1, CDK5 regulatory subunit associated protein-1 like 1; CDKN2, cyclin-dependent kinase inhibitor 2A; FTO, fat mass and obesity-associated; HHEX, haematopoietically expressed homeobox; IDE, insulin-degrading enzyme; IGF2BP2, insulin-like growth factor 2 mRNA-binding protein 2; KCNJ11, potassium inwardly-rectifying channel, subfamily J, member 11; PPARG, peroxisome proliferator-activated receptor-g gene; SLC30A8, solute carrier family 30 (zinc transporter), member 8; TCF2, transcription factor 2, hepatic; TCF7L2, transcription factor 7-like 2 (T-cell specific, HMg-box); WSF1 Wolfram syndrome 1.

PED/PEA-15 (Phosphoprotein Enriched in Diabetes / Phosphoprotein Enriched in Astrocytes of 15 KDa)

PED/PEA-15 identification

Some years ago, during a differential display-based study carried out in our lab to identify genes whose expression was altered in type 2 DM, the protein kinase C (PKC) substrate PEA-15 (Phosphoprotein Enriched in Astrocytes 15) was found overexpressed in fibroblast, skeletal muscle and adipose tissue from type 2 diabetics compared to healthy subjects. Given its overexpression in type 2 DM, the PEA-15 gene was re-named PED which stands for Phosphoprotein Enriched in Diabetes [7].

Much more recently, the PED overexpression finding has been replicated in a different population of type 2 diabetics and in a population of healthy subjects at high risk of developing diabetes, such as the first degree relatives of type 2 diabetics (FDR). Furthermore, in these same subjects, PED expression levels inversely correlate with insulin sensitivity in skeletal muscle [8]. These last evidences suggest that PED/PEA-15 may contribute to the early appearance of insulin resistance in healthy individuals at high risk of diabetes.

PED/PEA-15

PED/PEA-15 was originally identified as a major astrocytes phosphoprotein and it was found to be widely expressed in different tissues and highly conserved among mammals. PED/PEA-15 is a 15KDa cytosolic protein whose gene maps on chromosome 1q21-22 [7]. It is a highly regulated protein with two major phosphorylation sites on Ser104 and Ser116. Indeed, PED/PEA-15 is phosphorylated at Ser104 by the protein kinase C (PKC) and at Ser116 by the calcium-calmodulin kinase II (CAMKII) and by Akt/PKB [9; 10]. Moreover, it presents at the N-

terminus the protein-protein interaction domain DED (Death Effector Domain) and a NES (Nuclear Export Signal), that localizes the protein prevalently into the cytosol (Fig.2).

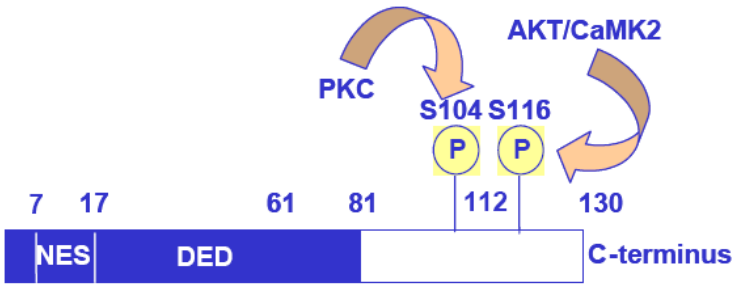

Figure 2 – Schematic representation of the PED/PEA-15 protein

Several studies in cultured cells and in rodent tissues have revealed that PED/PEA-15 is a multifunctional protein. I) It regulates multiple cellular function by binding to distinct components of major intracellular transduction pathways [11-13]. These include PLD1, ERK1/2, Akt/PKB and RSK2. II) It has been shown to exert antiapoptotic action by distinct mechanisms. Firstly, PED/PEA-15 inhibits the formation of the death-inducing signalling complex (DISC) and caspase 3 activation by different apoptotic cytokines including FAS ligand, tumour necrosis factor alpha, and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) [14; 15]. At least in part, this action is accomplished through the DED of PED/PEA-15 upon PED/PEA-15 recruitment to the DISC. Secondly, PED/PEA-15 inhibits the induction of different stress-activated protein kinases (SAPKs) triggered by growth factor deprivation, hydrogen peroxide and anisomycin [16]. This action of PED/PEA-15 is exerted by the blocking of an upstream event in the SAPK activation cascade and requires the interaction of PED/PEA-15 with ERK1/2. Thirdly, PED/PEA-15 modulates apoptosis upon UVC exposure in a dose-dependent fashion. Indeed, at least in part, apoptosis following

Omi/HtrA2 mitochondrial release is mediated by reduction in PED/PEA-15 cellular levels [17]. Thus, the ability of Omi/HtrA2 to relieve XIAP inhibition on caspases is modulated by the relative levels of Omi/HtrA2 and PED/PEA-15.

III) It plays an important role in tumour development and sensitivity to antineoplastic agents [18]. Indeed, the expression levels of PED/PEA-15 control caspase 3 function and epidermal cell apoptosis in vivo and determine susceptibility to skin tumour development. IV) PED/PEA-15 also binds to and increases the stability of the phospholipase D1, enhancing its activity and controlling important mechanisms in cell metabolism [11]. In cultured muscle and adipose cells and in tissues from transgenic mice, high levels of PED/PEA-15 impair insulin-stimulated GLUT4 translocation to the plasma membrane and glucose transport suggesting that PED/PEA-15 overexpression may contribute to insulin resistance in type 2 DM [19; 20]. Moreover, other studies have demonstrated that PED/PEA-15-induced resistance to insulin action on glucose disposal is accompanied by PLD-dependent activation of the classical protein kinase C isoform PKCalpha. In turn, the induction of PKCalpha by PED/PEA-15 prevents subsequent activation of the atypical PKCzeta by insulin [19]. Thus, in muscle and adipose cells, PED/PEA-15 generates resistance to insulin action on glucose disposal by impairing normal regulation of PKCzeta (Fig.3).

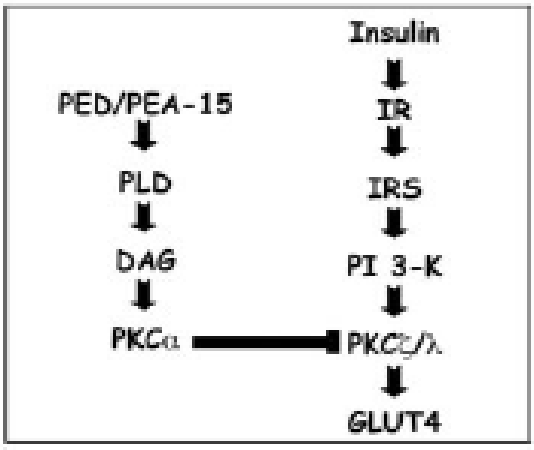

Figure 3 – Proposed mechanism of PED/PEA-15 action on insulin-stimulated glucose transport.

Transgenic mice ubiquitously overexpressing ped/pea-15 (Tg-PED) at levels comparable to those found in many type 2 diabetics, exhibited mildly elevated random-fed blood glucose levels and decreased glucose tolerance. These mice develop diabetes only when they are fed with a 60% fat diet [20], indicating that ped/pea-15 overexpression alone is able to generate a metabolic background prone to diabetes development. Much more recently a beta-cell specific ped/pea-15 transgenic animal has been generated to further elucidate the role of ped/pea-15 in the glucose-induced insulin secretion. It has been shown that ped/pea-15 overexpression in the beta-cell is able to impair glucose-induced insulin secretion by restraining potassium channel expression [21].

REGULATION OF GENE EXPRESSION BY THE SUPERFAMILY OF HORMONE NUCLEAR RECEPTORS

The nuclear receptor superfamily includes receptors for hormones such as steroids, thyroid hormone, retinoic acid and vitamin D, as well as a large number of homologous proteins with no ligand identified, termed the orphan receptors [22]. Many non-steroid hormone receptors, including

the thyroid hormone receptor (TR) and retinoic acid receptor (RAR), function as heterodimers with the retinoid X receptor (RXR). The receptors bind to specific DNA sequences in the promoter regions of target genes and activate transcription in the presence of cognate ligand. In addition, receptors such as TR and RAR repress transcription in the absence of ligand. It is important to point out that this refers to repression of basal transcription, rather than inhibition of activated transcription, as is associated with hormone-induced negative regulation.

Biological/Pathological significance of repression by nuclear receptors

The ability of nuclear receptors to bind and repress target genes in the absence of ligand serves to amplify the activating effect of hormonal signals. There is abundant and long-standing evidence for a biological role for the hormonal activation of nuclear receptors, but only recently has the importance of basal repression been appreciated. Initial clues came from the study of acute promyelocytic leukemia (APL). APL is associated with rearrangements of the gene encoding RAR-α (RARA), which produce abnormal fusion proteins that block myeloid differentiation. This differentiation block correlates with the ability of the fusion protein to repress transcription. Treatment with retinoic acid, which abolishes the RAR-mediated repression, relieves this block and results in complete remission of the disease in most patients, including those with the most common rearrangement involving the promyelocytic leukaemia gene (PML). However, patients in whom the RARA gene is fused to the promyelocytic leukemia zinc finger (PLZF) gene (ZNF145) are resistant to retinoic acid [23]. This correlates with an independent, retinoic acid resistant-repression function of PLZF [24-27]. The importance of the repression complex in leukemia is not restricted to APL. Acute myeloid leukemia (AML) caused by fusion of the AML

protein to the ETO (eight twenty one or MTG8) protein has also been linked to nuclear receptor repression mechanisms [28-30]. In addition, an oncogenic form of the TR, v-ErbA, contributes to avian erythroblastosis virus-induced leukemic transformation by repressing transcription of target genes [31]. Repression is also implicated in the syndrome of thyroid hormone (TH) resistance, which is caused by mutations in the ligand-binding domain (LBD) of the TR. The severity of the resistance correlates with the repression function of the mutant TR [32; 33].

Recent evidence also suggests a role for repression in normal thyroid physiology. The phenotype of mice lacking all known TRs is less severely altered than that of mice lacking TH [34]. A major difference between the two models is the presence of unoccupied receptor in the case of TH deficiency. This implies a role for the unliganded receptor in normal biology; for example, basal repression of target genes.

The Hepatocyte Nuclear Factor-4alpha and the Chicken Ovalbumin Upstream Promoter-Transcription Factor II

Hepatocyte Nuclear Factor-4alpha (HNF-4alpha) and the Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII) are two members of the steroid/thyroid superfamily of transcription factors involved in the control of glucose homeostasis [35-37]. Studies in mice in which the early lethal phenotype is circumvented have revealed that HNF-4alpha is essential for hepatocyte differentiation both at the morphological and the functional levels [38] and for accumulation of hepatic glycogen stores and generation of normal hepatic epithelium [39]. Point mutations in HNF-4alpha impair liver and pancreatic regulation of glucose homeostasis and cause Maturity Onset Diabetes of the Young type 1 (MODY1). More recently, genetic and biochemical evidence has

been generated indicating that HNF-4alpha may also have a role in the development of more common forms of type 2 diabetes [40-42].

Most of the promoter elements interacting with HNF-4alpha can also recognize the Chicken Ovalbumin Upstream Promoter Transcription Factors (COUP-TFs) [43-45] one of the most extensively studied orphan receptors. COUP-TFs regulate a number of biological processes including embryonic development [46] and neural cell fate determination [47]. COUP-TFs may also affect glucose homeostasis. Indeed, in vitro studies indicate that COUP-TFII, also termed Arp-1, regulates several genes involved in glucose and lipid metabolism including insulin gene expression in pancreatic beta-cells [48; 49]. Functionally, COUP-TFII has been identified as a transcriptional repressor of genes activated by HNF-4alpha. However, evidence is also present in the literature indicating that, at least in certain circumstances, COUP-TFII activates gene expression [50; 51]. The specific function of COUP-TFII likely depends upon the repertoire of coregulatory proteins interacting with COUP-TFII and HNF-4alpha in each specific context [52-60].

Hormone nuclear receptors and chromatin remodelling

Hormone nuclear receptors are ligand-activated transcription factors that regulate a vast array of biological processes by modulating expression of specific target genes [61; 62]. Their ability to modulate transcription depends on the recruitment of cofactors that remodel chromatin and the assembly of basal transcription machinery [63; 64].

Chromatin is the state in which DNA is packaged within the cell. The nucleosome is the fundamental unit of chromatin and it composed of an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped. The core histones are predominantly globular except for their N-terminal “tails”, which are unstructured. A

striking feature of histones, and particularly of their tails, is the large number and type of modified residues they possess (Fig.4).

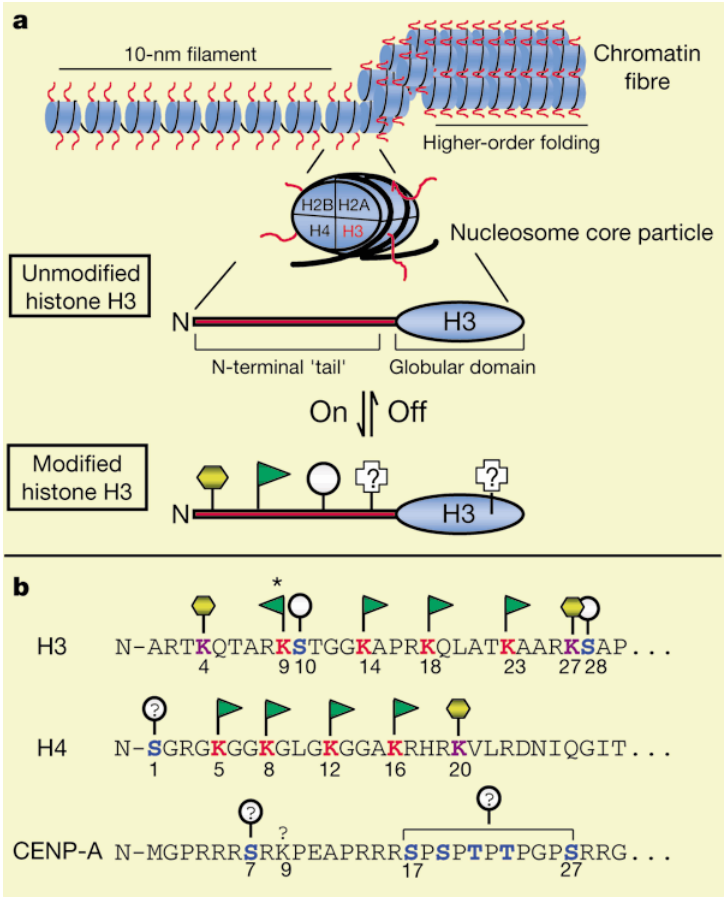

Figure 4 – Chromatin organization of the tail of histone H3 - a, General chromatin organization. Like other histone 'tails', the N terminus of H3 (red) represents a highly conserved domain that is likely to be exposed or extend outwards from the chromatin fibre. A number of distinct post-translational modifications are known to occur at the N terminus of H3 including acetylation (green flag), phosphorylation (grey circle) and methylation (yellow hexagon). Other modifications are known and may also occur in the globular domain. **b**, The N terminus of human H3 is shown in single-letter amino-acid code. For comparison, the N termini of human CENP-A, a centromere-specific H3 variant, and human H4, the nucleosomal partner to H3, are shown. Note the regular spacing of acetylatable lysines (red), and potential phosphorylation (blue) and methylation (purple) sites. The asterisk indicates the lysine residue in H3 that is known to be targeted for acetylation as well as for methylation; lysine 9 in CENP-A (bold) may also be chemically modified. The above depictions of chromatin structure and H3 are schematic; no attempt has been made to accurately portray these structures.

Histone modifications in chromatin, particularly histone methylation, play key roles in gene expression and are emerging as a visible new layer of gene expression regulation [65-67]. Depending upon the timing and chromosomal location, histone methylation can not only undergo

dynamic changes during gene transcription and cell division, but also remain semi-stable, well maintained, and somatically inheritable. Along with DNA methylation, histone methylation can contribute to epigenetic heritable changes in gene function that do not involve a local change in DNA sequence. It has been shown that changes in histone methylation follow specific patterns and encode informations during cell cycle changes and development [68]. Therefore, aberrant alterations in histone lysine methylation patterns that change chromatin structure could lead to dysregulated gene transcription and disease progression [69]. Together with histone methylation, the acetylation status of histone proteins alters gene transcription. Removal of acetyl groups from lysine residues results in histone methylation, compaction of chromatin and, hence, repression of gene transcription [70]. As such, transcriptionally active euchromatin is tipically hyperacetylated and hypomethylated, compared with inactive heterochromatin which tends to be hypoacetylated and hypermethylated [71]. Elucidating the biological and functional relevance of these post-translational histone modifications is crucial to our understanding of the role of chromatin in gene expression and disease.

Chromatin remodelling is achieved by enzymes that catalyses post translational modifications of histones and the shuttling of nucleosomes by ATP-dependent mechanisms. The histone modifications, which include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, deimination, ADP ribosylation and proline isomerization, lead alterations in the accessibility of chromatin and allow for regulated transcription of genes [72]. These modifications, which may take place sequencially or in combination are proposed to constitute a “histone code” [73-75] that dictates the transcription state of genes (Fig.5).

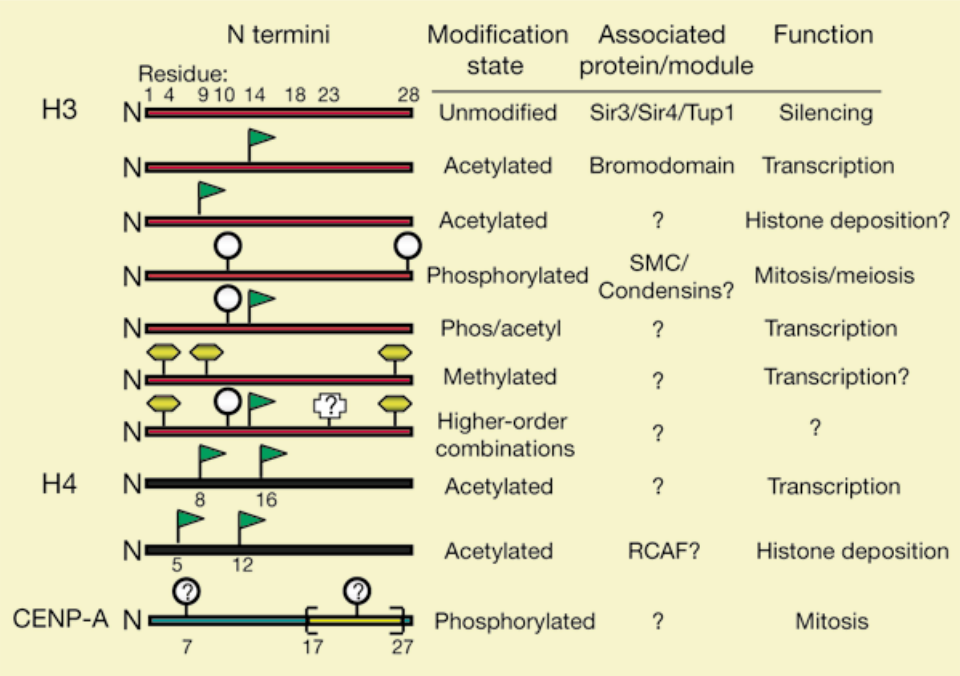

Figure 5 – The "Histone Code" hypothesis - Histone modifications occur at selected residues and some of the patterns shown have been closely linked to a biological event (for example, acetylation and transcription). Emerging evidence suggests that distinct H3 (red) and H4 (black) tail modifications act sequentially or in combination to regulate unique biological outcomes. How this hierarchy of multiple modifications extends (depicted as 'higher-order combinations') or how distinct combinatorial sets are established or maintained in localized regions of the chromatin fibre is not known. Relevant proteins or protein domains that are known to interact or associate with distinct modifications are indicated. The CENP-A tail domain (blue) might also be subjected to mitosis-related marks such as phosphorylation; the yellow bracket depicts a motif in which serines and threonines alternate with proline residues.

In addition to histone modifications, DNA methylation is a key epigenetic mechanism for gene silencing [76]. Methylation at cytosine residues of the dinucleotide sequence CpG is essential for animal development [77] and irregular methylation patterns lead to cancer [78].

The diverse biological processes regulated by nuclear receptors are attributed to differential recruitment of coactivators and corepressors that function as scaffolds for the recruitment of chromatin remodelling enzymes. In the absence of ligand, certain nuclear receptors recruit corepressors such as the Silencing Mediator of Retinoic acid and Thyroid hormone receptors (SMRT) and the Nuclear receptor corepressor (N-Cor) that bind repressive enzymes such as histone deacetylase enzymes

(HDAC) [79] or the histone methyl transferase (HMT) SUV39H1, which specifically methylates histone H3 at lysine 9 (H3K9) [80]. On the other hand, ligand bound nuclear receptors can also bind corepressors such RIP140 [81] and L-Cor [82] to actively repress gene expression (Fig.6).

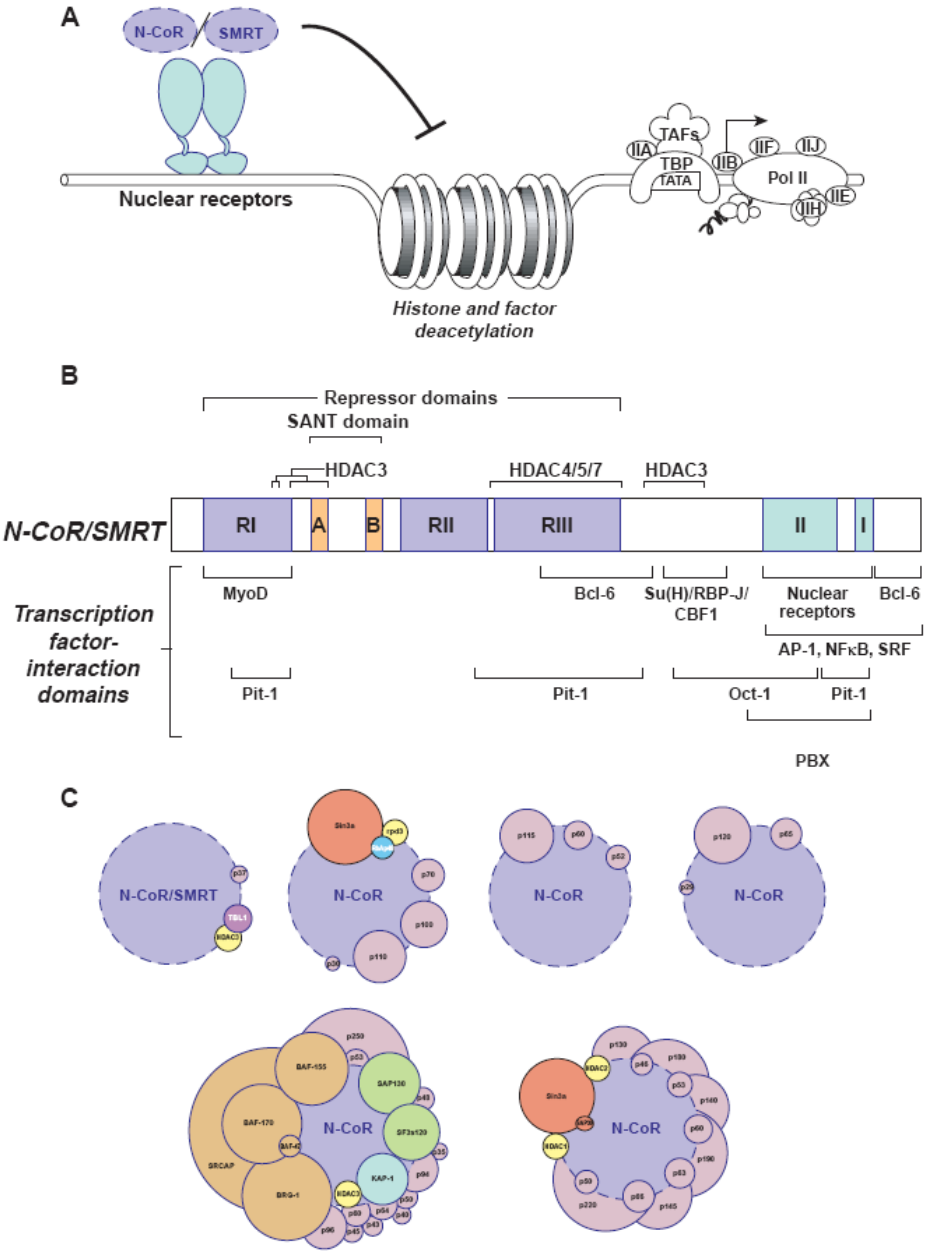

Fig. 6 – Nuclear Receptor Co-repressors (A) Transcriptional repression by nuclear receptors is regulated by recruitment of the co-repressors N-CoR and/or SMRT.
(B) The domains of N-CoR/SMRT. Repression domains (RI, RII, RIII) and SANT domains (A and B) are indicated, as are interaction domains for HDACs, nuclear receptors (I and II) and other transcription factors. (C) N-CoR–SMRT compexes. Biochemical purification techniques have revealed several different complexes recruited by N-CoR and/or SMRT [83-87]

AIM OF THE STUDY

PED/PEA-15 overexpression is a common feature of both type 2 diabetic individuals and healthy subjects at high risk of developing the disease, such as healthy first degree relatives of type 2 diabetic subjects.

Since no alteration in the DNA sequence of the *PED/PEA-15* gene has been found up to now which could account for the altered expression, the general aim of my research has been to discover new mechanisms involved in the regulation of its expression and potentially deregulated in diabetic conditions.

I have firstly cloned and characterized the promoter region of the human *PED/PEA-15* gene and obtained evidence that HNF-4alpha binds an HNF Response Element (HRE) on the promoter of the gene and represses its transcription.

Once established the role of the HNF-4alpha as an inhibitor of *PED/PEA-15* expression, at least in the liver, the aim of my further studies has been to clarify the molecular mechanisms by which HNF-4alpha exerts its action once bound to the HRE on the promoter of the *PED/PEA-15* gene.

To this aim, I have investigated: 1) whether HNF-4alpha acts “passively” on *PED/PEA-15* expression by competing with an activator for the binding to the promoter of the gene; 2) and whether, given its belonging to the superfamily of the hormone nuclear receptors, HNF-4alpha is able to recruit co-repressor complexes on *PED/PEA-15* promoter and inhibit its expression by modifying the accessibility of the core-promoter region to the basal transcriptional machinery.

Chapter 2
Materials and Methods

Materials. Media, sera, and antibiotics for cell culture and the Lipofectamine reagent were purchased from Invitrogen Ltd. (Paisley, United Kingdom). Goat polyclonal HNF-4alpha, SMRTe and Acetyl-Histone H3 K9/K14 (AcH3) and rabbit polyclonal COUP-TFII antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit monoclonal Di-methyl-H3-lysine 4 (H3K4) and mouse monoclonal Di-methyl-H3 lysine 9 (H3K9) were from AbCam (Cambridge – UK). PED/PEA-15 antibody has been previously described [7; 10]**.** The mouse monoclonal HA antibody was from Boehringer Mannheim. The Protein A- and G-Sepharose beads were from Pierce (Rockford, IL). The pCDNA3/HNF-4alpha expression vector, which contains the HNF-4alpha coding region, was a generous gift from Dr. Graeme Bell (Department of Medicine, University of Chicago, Chicago, Illinois). The pCMV6-XL5COUP-TFII expression plasmid and the pCMV6-XL5 vector were from OriGene Technologies, Inc. (Rockville, MD). Radiochemicals, Western blot and ECL reagents were purchased from Amersham Pharmacia Biotech. All other reagents were from Sigma (St. Louis, MO).

Plasmid constructs. A 2.0 Kb KpnI-XhoI genomic fragment containing the 5'-flanking region of the human *PED/PEA-15* gene was amplified (PCR) and subcloned between the corresponding restriction sites of the luciferase expression vector pGL3 Basic (Promega, Madison, WI). The resulting plasmid, termed pPED2000, was used as a template for PCR generation of progressively deleted fragments of the *PED/PEA-15* 5'-flanking region. These were also subcloned in the luciferase cloning vector. To minimize the possibility of introducing errors during the PCR,

the Expand Long Template PCR System (Roche Mannheim**)** was used and DNA was amplified according to the manufacturer's instructions. The identity and orientation of the PCR fragments were then assessed by restriction enzyme analysis and sequencing.

The HNF-4alpha binding site on *PED/PEA-15* promoter (TCATCCAAAGGTCA) was mutagenized by PCR to TCATCCCCCGGTCA. Two mutated inner primers (pPED477mutFor CGTGGTCATCCCCCGGTCAAAAG and pPED477mutRev CTTTTGACCGGGGGATGACCAGG) and two outer primers (Ped477 KpnI and Ped (XhoI) antisense – see table1) were used. Two mutated products were amplified using the pPED477 construct as a template and the Expand Long Template PCR System (Roche) to minimize the possibility of introducing errors during the PCR. In the first reaction the mutated inner sense primer was used together with the Ped (XhoI) antisense, while the Ped477 KpnI primer was used in the second reaction together with the mutated inner antisense. The mutant HRE was then reconstituted with the Expand Long Template PCR System (Roche) using the outer primers.

The mutant fragment (477mut) obtained was then inserted into the pGL3 Basic Vector and completely sequenced.

All siRNAs were chemically synthesized by Ambion (Austin, TX) as oligonucleotide duplexes. siRNA target sequences for SMRT were directed at regions common to both SMRTα and SMRTβ (panSMRT, 5'-GGGTATCATCACCG CTGTG-3' [88]. As nonspecific siRNA controls, an siRNA sequence targeting luciferase (5'-CGTACGCGGAATACTTC GA-3') was used.

Cell culture, transfections, RT-PCR, and Western Blot assay. Hela, and HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, Penicillin (200 IU/ml), Streptomycin (100 μg/ml), and 2% $_{L}$-glutamine in a humidified CO_2 incubator. Stable expression of the *HNF-4alpha* sh-RNA clone in HepG2 cells and wild type HNF-4alpha cDNA in Hela cells was achieved as reported by Condorelli et al (16). Transient transfection of plasmid DNAs in Hela, and HepG2 cells were carried out by the calcium phosphate coprecipitation method [89]. Briefly, the cells were plated in 60-mm dishes prior to transfection at a confluence of 1 x 10^5 cells/dish. 3 μg of the indicated *PED/PEA-15* promoter-luciferase construct and 1 μg of the pRSVβ-gal vector (to correct for the variable transfection efficiencies) were then added.

To examine the effect of HNF-4alpha and COUP-TFII on *PED/PEA-15* promoter, Hela and HepG2 cells were cotransfected with 2 μg of *PED/PEA-15* promoter-luciferase together with different amounts of HNF-4alpha and COUP-TFII expression vectors. Total DNA content (up to 4 μg/plate) was normalized to the empty vector devoid of HNF-4alpha and COUP-TFII coding sequence. 48 h after transfection, the cells were harvested and lysed as described previously [60]. Luciferase activity was measured by a commercial luciferase assay kit (Promega). Values were normalized for β-galactosidase. Statistical significance was evaluated by t-test analysis.

Total RNAs were prepared by extraction with the RNeasy Kit (Qiagen Sciences) according to the manufacturer's instructions. For Real-Time RT-PCR analysis, 1 μg RNA was reverse-transcribed using the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Reactions were performed using Platinum SYBR Green qPCR Super-UDG in a iCycler IQ multicolor Real Time PCR Detection System

(Biorad, Hercules, CA). All reactions were performed in triplicate, and β-actin was used as internal standard.

For Western blot and Co-immunoprecipitation analysis, cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_2P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml aprotinin) for 2 h at 4°C. Cell lysates were clarified by centrifugation at 5,000 x *g* for 20 min, separated by SDS-PAGE (or firstly immunoprecipitated), and transferred into 0.45-µm Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with primary and secondary antibodies, immmunoreactive bands were detected by ECL according to the manufacturer's instructions.

shRNA-mediated knockdown of HNF-4alpha To interfere with endogenous HNF-4alpha expression in HepG2 cells, we use short-hairpin RNAs with the following sequences: clone a (Cl.a) CCGGCCATCACCAAGCAGGAAGTTACTCGAGTAA CTTCCTGCTTGGTGATGGTTTTT; clone b (Cl.b) CCGGACCACCCTGGAATTT GAGAATCTCGAGATTCTCAAATTCCAGGGTGGTTTTTT. The cells were transfected with 1 µg of each clones using the Lipofectamine reagent according to manufacturer's recommendations and analyzed 48 h after transfection.

5-aza-2'-deoxycytidine and trichostatin A treatment HepG2 cells were seeded in 10 cm dishes at a density of 1 X 10^6 cells 1 day before the drug treatment. The cells were treated with 10 mM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma, St Louis, MO) for 24 h then harvested.

Another culture of cells was treated with 330 nM trichostatin A (TSA; Sigma) for 1 day. To test the combined effect of 5-aza-dC and TSA, cells were treated with 10 mM 5-aza-dC and 330 nM TSA for 24h.
Total RNA and chromatin were prepared and tested for restoration of PED/PEA-15 and HNF-4alpha expression by real-time RT–PCR and HNF-4alpha occupancy and PED/PEA-15 promoter modifications by ChIP assays.

FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) assay. The cross-linking solution, containing 1% formaldehyde, was added directly to cell culture media. The fixation proceeded for 10 min and was stopped by the addition of glycine to a final concentration of 125 mM. cells were rinsed twice with cold PBS plus 1 mM PMSF, and then (Hela and HepG2) scraped. Cells were collected by centrifugation at 800 X g for 5 min at 4°C. Cells were swelled in cold cell lysis buffer containing 5 mM piperazine-N,N'-bis (2ethanesulfonic acid) (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF and inhibitors cocktail (Sigma) and incubated on ice for 10 min. Nuclei were precipitated by microcentrifugation at 2000 X g for 5 min at 4°C, resuspended in nuclear lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.8% sodium dodecyl sulphate (SDS), 1 mM PMSF and inhibitors cocktail (Sigma), and then incubated on ice for 10 min. Samples were broken by sonication into chromatin fragments of an average length of 500/1000 bp and then microcentrifuged at 16,000 X g for 10 min at 4°C. Protein-free DNA was then extracted with Phenol-Chloroform-Isoamyl alcohol (25:24:1) and precipitated. The pellets were resuspended in 30 μl of TE and analyzed by PCR using specific primers for the analyzed regions. The input sample (obtained by a step of reversal cross-link, deproteinization

and extraction as described below) was resuspended in 30 μl of TE and diluted 1:10 before PCR.

Chromatin Immuno-precipitation (ChIP) and ReChIP assay. The cross-linking solution, containing 1% formaldehyde, was added directly to cell culture media. The fixation proceeded for 10 min and was stopped by the addition of glycine to a final concentration of 125 mM. Primary human hepatocytes (Lonza Walkersville), Hela and HepG2 cells were rinsed twice with cold PBS plus 1 mM PMSF, and then (Hela and HepG2) scraped. Cells were collected by centrifugation at 800 X g for 5 min at 4°C. Cells were swelled in cold cell lysis buffer containing 5 mM piperazine-N,N'-bis (2ethanesulfonic acid) (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF and inhibitors cocktail (Sigma) and incubated on ice for 10 min. Nuclei were precipitated by microcentrifugation at 2000 X g for 5 min at 4°C, resuspended in nuclear lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.8% sodium dodecyl sulphate (SDS), 1 mM PMSF and inhibitors cocktail (Sigma), and then incubated on ice for 10 min. Samples were broken by sonication into chromatin fragments of an average length of 500/1000 bp and then microcentrifuged at 16,000 X g for 10 min at 4°C. The sonicated cell supernatant was diluted 8-fold in ChIP Dilution Buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), and 167 mM NaCl, and precleared by adding Salmon Sperm and conjugating protein at equimolar concentration for 90 min at 4°C. Precleared chromatin from 1 X 10^6 cells was incubated with 1 μg of polyclonal antibody or no antibody and rotated at 4°C for 16h. Immunoprecipitates were washed five times with RIPA buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM PMSF; twice with LiCl buffer containing 0.25 M LiCl,

1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), and then three times with TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA). Before the first wash, the supernatant from the reaction lacking primary antibody was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Immunoprecipitates were eluted by adding 1% SDS, 0.1 M $NaHCO_3$ and reverse cross-linked by addition of NaCl to a final concentration of 200 mM and by heating at 65°C for at least 4h. Recovered material was treated with proteinase K, extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated. The pellets were resuspended in 30 µl of TE and analyzed by PCR using specific primers for the analyzed regions. The input sample was resuspended in 30 µl of TE and diluted 1:10 before PCR.

For ReChIP assay, immunoprecipitates with the first antibody were eluted in 50 ml of DTT 10 mM, diluted 10-fold in ChIP Dilution Buffer supplemented with protease inhibitors, and immunoprecipitated with the second antibody. Following immunoprecipitation, samples are processed as described above for ChIP assay and eluted DNA amplified by PCR with specific oligos.

MNase (Micrococcal Nuclease) Protection assay. Nuclei were isolated from ~10^8 Hela, HelaHNF4, HepG2 and HepG2sh cells. Isolated nuclei were suspended in 1 ml of wash buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, and 8.5% sucrose) and digested with 120U of micrococcal nuclease (MNase; Worthington Biochemical, Freehold, NJ) for 30 min at 37°C. Reactions were stopped by the addition of 100 µl of 5 M NaCl and 100 µl of stop solution (10% SDS, 125 mM EDTA, and 1 mg/ml proteinase K) at 50°C for 3 h. Genomic DNAs were separated on a 1.5% agarose gel. The bands

corresponding to mononucleosome (150 bp) were cut with a clean blade, and DNAs were isolated from the excised gel slice using the GFX PCR purification Kit (GE Healthcare). The purified DNA was quantified and identified on an agarose gel again and subsequently amplified by PCR using the N1F/R, N2F/R and Ex-1 F/R couples of primers.

CHAPTER 3

Molecular cloning and characterization of the human PED/PEA-15 promoter reveals its antagonistic regulation by HNF-4alpha and COUP-TFII

RESULTS

Localization of the promoter activity within the 5'-flanking region of the PED/PEA-15 gene.

To verify whether the proximal 5'-flanking region of the *PED/PEA-15* gene contains a functional promoter, reporter gene assays were performed in human embryonic kidney (HEK 293) and cervical carcinoma (Hela) cell lines. A 2 Kb PCR product, containing the proximal 5'-flanking region, the transcription start site and 58 bp of exon 1, was cloned upstream to a promoterless luciferase reporter gene (fragment -1942/+58, pPED2000). Three independent clones were tested showing similar promoter activity both in the Hela and HEK 293 cells (data not shown). Next, the pPED2000 plasmid was used as a template to generate progressive deletion fragments of the *PED/PEA-15* 5'-flanking region, which were analyzed for promoter activity (Fig.7). The constructs were transfected in Hela and HEK 293 cell lines and luciferase activity was measured after 48h. Significant transcriptional activity compared to the basal luciferase activity was obtained in both cell lines with all of the deletion constructs encompassing the *PED/PEA-15* promoter region -1942/-39. Indeed, the shortest -39/+58 fragment still has a significantly higher luciferase activity compared to the empty vector, suggesting that all the basal promoter activity is contained in the very proximal 5'-flanking region. Since luciferase activity significantly declines from -230, it is possible that the minimal promoter is located within this sequence (Fig.7). Truncation of promoter at an upstream region between -1042 and

-542 and between -419 and -309 produced a decrease in the *PED/PEA-15* transcriptional activity, indicating that these regions contain positive regulatory elements of the basal promoter function. A further deletion between -309 and -230 revealed the presence of a potential transcription silencer.

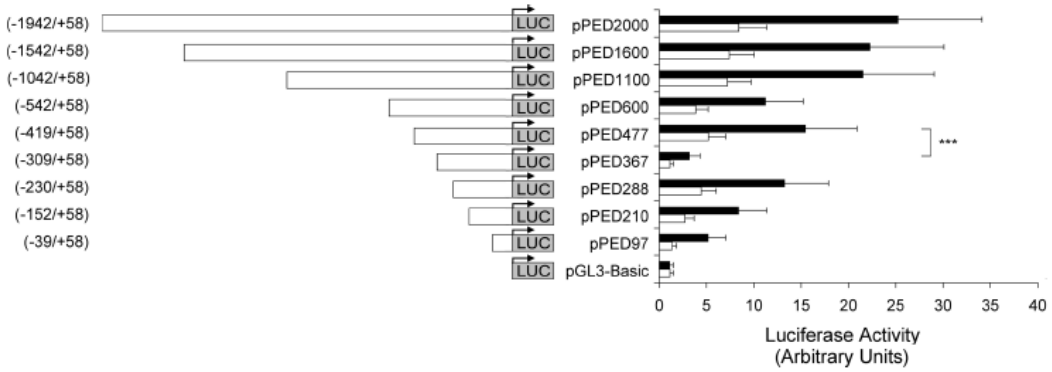

Figure 7 – Characterization of the promoter activity of the *PED/PEA-15* 5' flanking region. The *PED/PEA-15* 5' flanking fragments were cloned upstream to a promoterless luciferase reporter gene in the pGL3 Basic vector as described under Materials and Methods. Hela (black bars) and HEK293 cells (grey bars) were then cotransfected with 3 mg of the construct DNAs (or 3 mg of the promoterless pGL3 Basic vector DNA) and 1 mg of the pRSVb-gal vector DNA. Luciferase activity was assayed as described under Materials and Methods and is presented as increase above the activity measured with the pGL3 Basic vector. The results are presented as the means (normalized for b-galactosidase activity) ± SD of four independent experiments each performed in quadruplicate. Asterisks denote statistically significant differences ($p < 0.001$).

Regulation of the PED/PEA-15 transcriptional activity by HNF-4alpha.

In silico analysis of the *PED/PEA-15* promoter region revealed the presence of a 5'-GTCATCCAAAGGTCAAA-3' sequence located between the -419/+58 and -309/+58 fragments. This sequence closely resembles the HNF-4alpha responsive element (HRE) (44-45), suggesting the presence of an HNF-4alpha binding site in the promoter of the *PED/PEA-15* gene. To address the role of HNF-4alpha in *PED/PEA-15* promoter transcriptional activity, we co-transfected the -419/+58 *PED/PEA-15* promoter-luciferase construct (pPED477) together with an HNF-4alpha expression vector, (pCDNA3/HNF-4alpha) into Hela cells, which feature low levels of the endogenous HNF-4alpha. As shown in

Fig.8A, HNF-4alpha reduces the reporter gene activity in a dose-dependent manner. This effect is specific, as it is lost when the cells are transfected with a vector containing the mutagenized HNF-4alpha binding sequence (pPED477mut) and a 5' deletion construct lacking the HNF-4alpha site (pPED210) (Fig.8B).

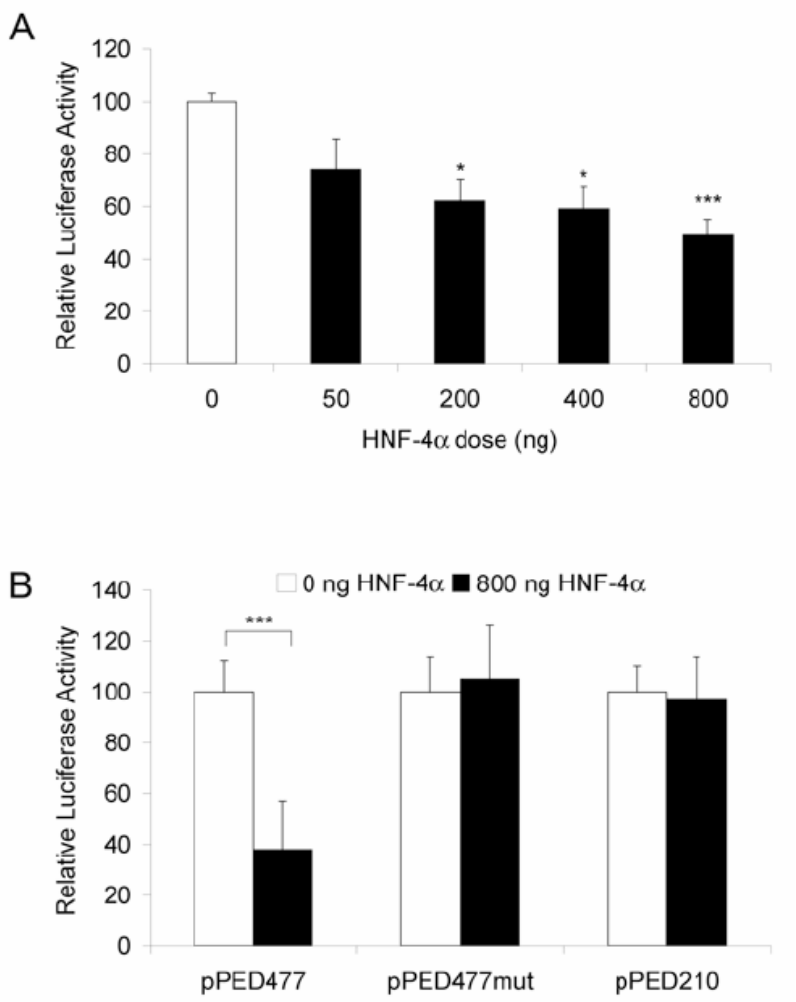

Figure 8 – Regulation of PED/PEA-15 promoter activity by HNF-4alpha. (A) Hela cells were co-transfected with 2 μg of the pPED477 *PED/PEA-15* promoter-luciferase construct and the indicated amounts of the pCDNA3/HNF-4alpha expression vector. (B) Alternatively, the cells were co-transfected with the pPED477, the pPED477mut or the pPED210 *PED/PEA-15* promoter luciferase constructs and 0.8 μg of the pCDNA3/HNF-4alpha expression vector. Luciferase activities were normalized for those of β-galactosidase and are presented as the means ± SD of four independent experiments each performed in quadruplicate. Asterisks denote statistically significant differences (* $p < 0.05$; *** $p < 0.001$).

Consistent with these luciferase assays, Real Time PCR assay on total RNA extracted from HNF-4alpha transfected Hela cells confirmed a 2-fold decrease in *PED/PEA-15* expression levels (Fig.9A). Same analysis was performed by Western blot with similar results: as shown in Fig.9B, the levels of PED/PEA-15 protein decreases in HNF-4alpha transfected Hela cells. Moreover, we observed an inverse correlation between PED/PEA-15 and HNF-4alpha protein levels in different cell types. In fact, HepG2 cells present well detectable levels of endogenous HNF-4alpha and low levels of PED/PEA-15, while the opposite is observed in the Hela and HEK 293 cells both of which show low levels of HNF-4alpha and high levels of PED/PEA-15 (Fig.9C).

Overall, our results indicate that HNF-4alpha negatively regulates *PED/PEA-15* gene expression.

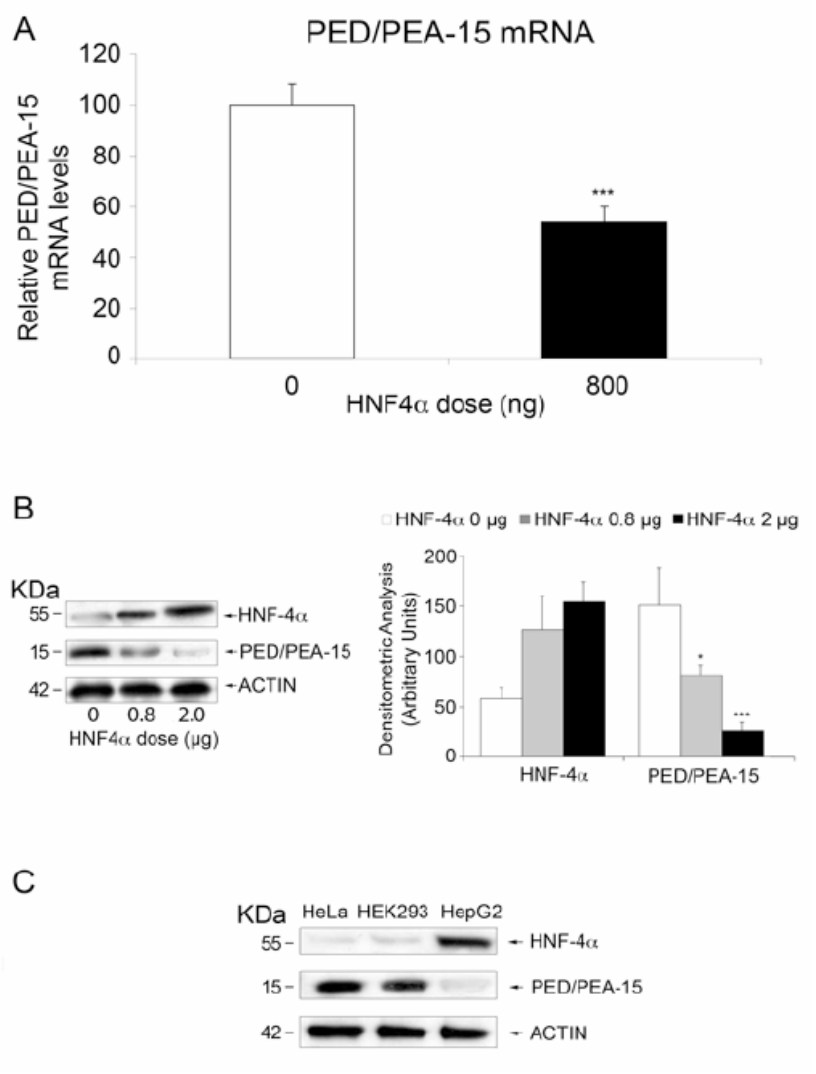

Figure 9 – Correlation between PED/PEA-15 and HNF-4alpha levels. (A) Hela cells were transfected with 0.8 μg of the pCDNA3/HNF-4alpha expression vector. *PED/PEA-15* mRNA levels were then quantitated by RT-PCR. Data were normalized for β-actin mRNA and are expressed as % decrease vs. control (untransfected cells). (B) Hela cells were transfected with the indicated amounts of the pCDNA3/HNF-4alpha vector DNA. The cells were then solubilized and Western blotted with PED/PEA-15 antibodies followed by reblotting with HNF-4alpha antibodies. (C) Lysates from Hela, HEK293 and HepG2 cells (40 μg protein/sample) were analyzed by Western blotting with PED/PEA-15 antibodies followed by reblotting with HNF-4alpha and actin antibodies. All filters were revealed by ECL and autoradiography and quantitated by laser densitometry of the autoradiographs. Bars represent the means ± SD of three (B) and four (A) independent experiments. Asterisks denote statistically significant differences (* $p < 0.05$; *** $p < 0.001$). Representative blots are also shown.

HNF-4alpha binds the cis-acting element at position -335 to -320 in the PED/PEA-15 promoter.

We next tested the ability of HNF-4alpha to bind the putative binding sequence in the *PED/PEA-15* gene promoter. EMSA was performed using a probe containing the putative HNF-4alpha-binding site, designated HNF-4alpha RE element (Fig.10A). Whole cell extracts were obtained from Hela cells transfected either with an HNF-4alpha expression plasmid or a control vector. Incubation of HNF-4alpha-transfected Hela cell extracts with the radiolabeled probe resulted in a strong band that was absent when the cells were transfected with the empty vector (lanes 2 and 1). Competition with unlabeled HNF-4alpha RE oligonucleotide abolished this band (lane 3). In contrast, increasing amounts of the mutated HNF-4alpha RE probe added to the binding reactions competed for HNF-4alpha binding to the probe encompassing the wild-type sequence much less efficiently (lanes 4-6). These data indicate that in vitro HNF-4alpha binds to the *PED/PEA-15* HNF-4alpha RE site at – 335 to –320 position.

Occupancy of the *PED/PEA-15* promoter by HNF-4alpha in living cells was further analyzed by Chromatin Immunoprecipitation (ChIP) experiment. Wild-type HepG2 cells which show well detectable levels of this transcription factor, were used. Chromatin DNA was precipitated using either the anti-HNF-4alpha or anti-HA antibodies as negative control, and the sequence encompassing the HNF-4alpha consensus binding site was amplified using specific primers. As shown in Fig.10B, precipitation with HNF-4alpha (lane 2a) but not HA (lane 3a) antibodies enabled amplification, indicating occupation of the *PED/PEA-15* promoter by HNF-4alpha. As a positive control, a 145-bp fragment containing the HNF-4alpha response element of the *UGT1A9* promoter was also amplified from genomic DNA precipitated with the HNF-4alpha

antibody (lane 2b) [90]. Consistently, PCR amplification with oligonucleotides for *β-GLOBIN* (negative control) did not show any signal. Same results were also obtained with primary human hepatocytes (Fig.10B).

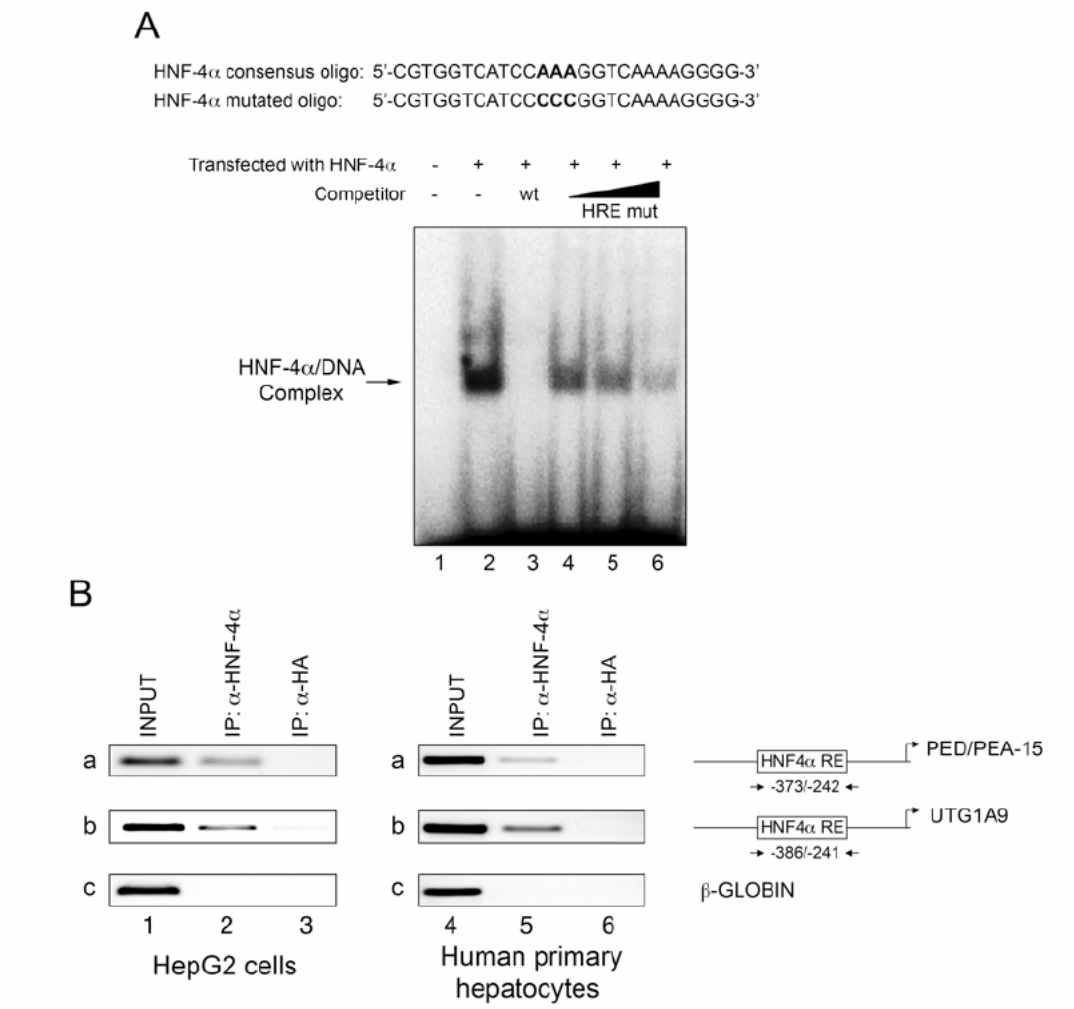

Figure 10 – HNF-4alpha binding to the PED/PEA-15 gene promoter. (A) EMSA.Whole cell extracts from Hela cells transfected with either the empty plasmid (lane 1) or the HNF-4alpha expression vector (lanes 2-6) were incubated with the ^{32}P-labelled HNF-4alpha RE probe. Incubation occurred in the absence (lanes 1-2) or the presence of either a 4-fold molar excess of unlabelled HNF-4alpha RE probe (HREwt; lane 3) or 2-, 4-, 10-fold molar excess of unlabelled HNF-4alpha RE mutated oligonucleotides (HRE mut; lanes 4-6). Proteins were separated on a non-denaturing polyacrylamide gel and revealed by autoradiography. The autoradiograph shown is representative of four independent experiments. (B) ChIP assay. Soluble chromatin was prepared from HepG2 cells and human primary hepatocytes as described under Materials and Methods and immunoprecipitated with either HNF-4alpha (lanes 2 and 5) or HA antibodies (lanes 3 and 6). Total (INPUT; lanes 1 and 4) and

immunprecipitated DNAs were then amplified using primer pairs covering HNF-4alpha RE on the *PED/PEA-15* (a) and the *UGT1A9* promoters (b; positive control) or the *β-GLOBIN* (c; negative control). The photographs shown are each representative of three independent experiments.

To further assess the direct role of HNF-4alpha on *PED/PEA-15* expression, we used an RNAi-mediated approach. Transfection of HepG2 cells with HNF-4alpha specific sh-RNA clones caused a decrease of *HNF-4alpha* mRNA to 20% of the endogenous levels (Fig.11A). In the same cells, a parallel increase of *PED/PEA-15* mRNA and protein levels were observed (Fig.11B and C), supporting the important role of HNF-4alpha in controlling *PED/PEA-15* expression.

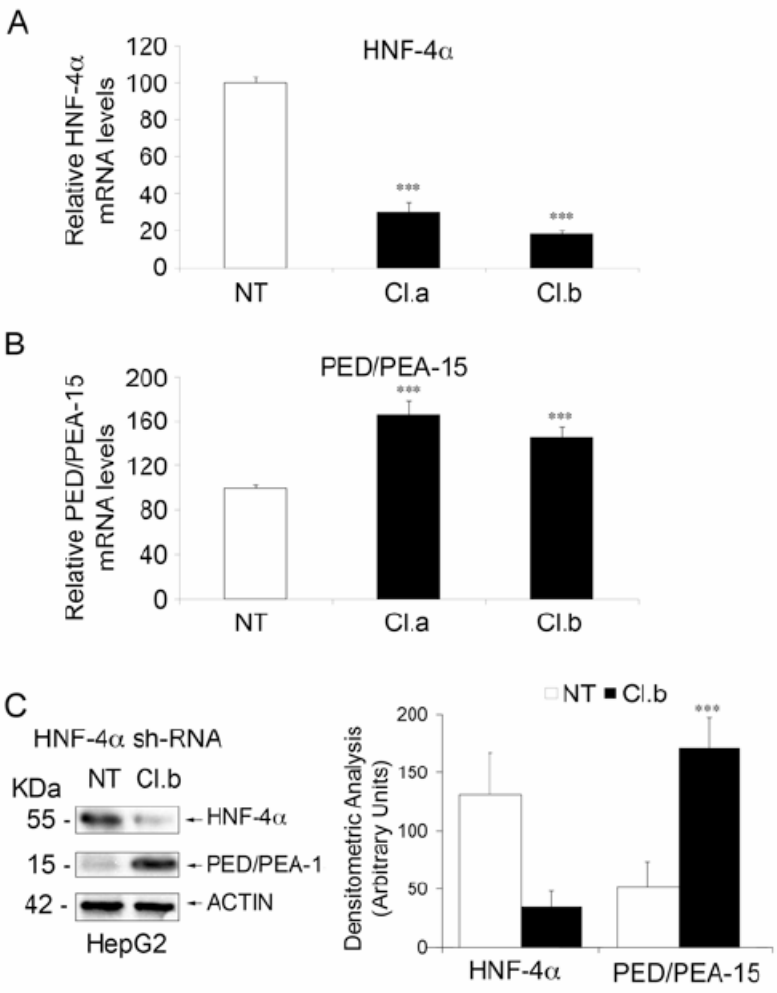

Figure 11 – The effect of RNAi-mediated silencing of HNF-4alpha on *PED/PEA-15* gene expression in HepG2 cells. HepG2 cells were transiently transfected with two HNF-4alpha-specific shRNA clones (Cl. a, b). 48 h upon transfection, total RNAs were extracted form transfected and non-transfected cells (NT). *HNF-4alpha* (A) and

PED/PEA-15 (B) mRNA levels were then quantitated by RT-PCR. Bars represent values normalized for *β-ACTIN* mRNA. (C) HepG2 cells were transiently transfected with the HNF-4alpha shRNA (Cl.b). After 48 h, transfected and non transfected cells were solubilized and lysates Western blotted with antibodies toward HNF-4alpha, PED/PEA-15 or β-ACTIN. Filters were revealed by ECL and autoradiography, followed by densitometric analysis of the autoradiographs. Bars represent the means ± SD of four (A,B) and three (C) independent experiments. Asterisks denote statistically significant differences. A representative blot is shown in the left panel of (C).

PED/PEA-15 gene transcription is activated by COUP-TFII.

COUP-TF antagonizes HNF-4alpha-dependent gene expression [44; 45; 91-93]. We have therefore hypothesized that COUP-TFs may also regulate *PED/PEA-15* transcription. To this aim, the pPED477 luciferase construct containing the HRE was transfected in Hela cells together with the COUP-TFII expression vector (pCMV6-XL5COUP-TFII). As shown in Fig.12A, COUP-TFII produced an activation of the reporter gene in a dose dependent manner (Fig.12A). This effect did not occur in cells transfected with a pPED477mut containing the mutagenized HNF-4alpha binding site and the 5' deletion construct lacking this site (pPED210) (Fig.12B), indicating that COUP-TFII activates the human *PED/PEA-15* promoter through the HNF-4alpha-responsive element. Real Time PCR assay and western blot analysis on COUP-TFII transfected Hela cells also confirmed the increase in *PED/PEA-15* expression level (Fig.12C and D). As shown in Fig.12E, COUP-TFII specifically bound the HRE on *PED/PEA-15* promoter in native Hela cells. At variance, COUP-TFII binding to the HRE was almost undetectable in the HepG2 cell lines which express HNF-4alphą at higher levels.

These data clearly indicate that COUP-TFII is involved in the regulation of Ped/Pea-15 expression and, in particular, it activates Ped/Pea-15 expression by binding the HNF-4alpha responsive element on its promoter.

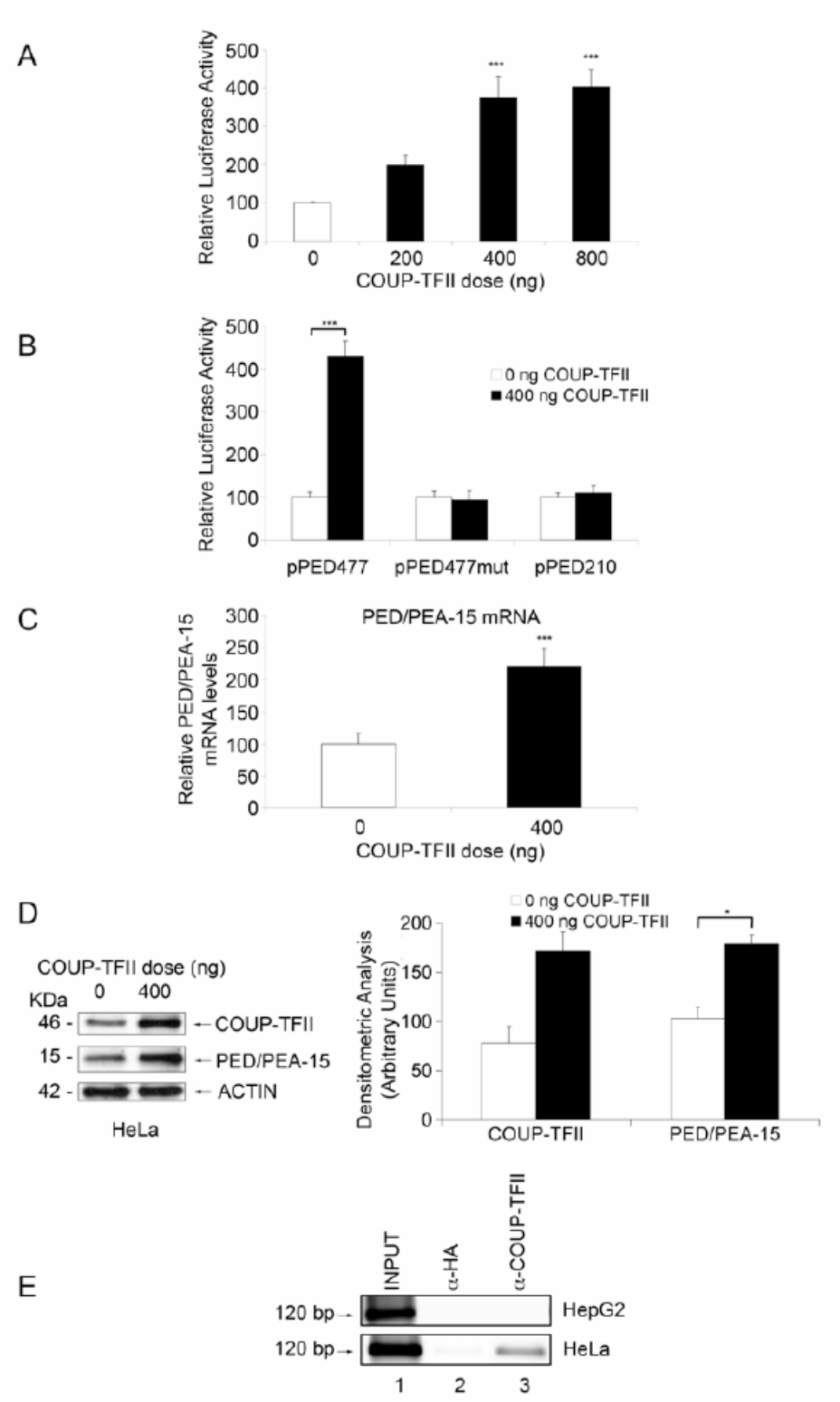

Figure 12 – COUP-TFII action on PED/PEA-15 expression. (A) Hela cells were transiently transfected with 2 μg of the pPED477 *PED/PEA-15* promoter-luciferase construct, the indicated amounts of the pCMV6-XL5COUP-TFII plasmid and 1 mg of the pRSV-bgal plasmid (transfection efficiency control). Alternatively (B), the cells were transfected with the pPED477, the pPED477mut or the pPED210 *PED/PEA-15* promoter luciferase constructs, together with 0.4 mg of the COUP-TFII expression vector and 1 mg of the pRSV-bgal expression plasmid. Luciferase activity was assayed as described under Materials and Methods and normalized for b-galactosidase. (C) Hela cells were transfected with 0.4 mg of the COUP-TFII

expression vector. Upon 48 h, *PED/PEA-15* mRNA levels were quantitated by RT-PCR and normalized for *b-ACTIN* mRNA. Bars represent the means ± SD of three (A,B) and four (C) independent experiments, each in quadruplicate. All data are expressed as increases above values in control cells. (D) Hela cells were transfected with 0.4 mg of the COUP-TFII expression plasmid, solubilized and proteins (40 mg/sample) were separated by PAGE and analyzed by Western blotting with COUP-TFII and PED/PEA-15 antibodies, as indicated. Filters were revealed by ECL and autoradiography and autoradiographs subjected to densitometry. Bars represent the means ± SD of three independent experiments. A representative experiment is shown in the left panel. Asterisks denote statistically significant differences (* $p < 0.05$; ***$p < 0.001$). (E) HepG2 and Hela cells were cross-linked with 1% formaldehyde and subjected to Chip assays using COUP-TFII (lanes 3) and HA antibodies (lanes 2; negative control). Total (lane 1; INPUT) and immunoprecipitated DNAs were amplified using primers covering the HNF-4a RE in the *PED/PEA-15* gene. The photographs shown are representative of at least three independent experiments.

COUP-TFII opposes HNF-4alpha repression of PED/PEA-15 gene expression.

Since HNF-4alpha and COUP-TFII appear to bind the same response element on the promoter of *PED/PEA-15* gene, we hypothesized that these two proteins compete with each other for binding. Therefore, transient transfection assays were performed with the pPED477 reporter construct and various combinations of HNF-4alpha and COUP-TFII expression vectors. As shown in Fig.13A, HNF-4alpha repression of the pPED477 construct expression in Hela cells can be completely overcome by increasing amounts of COUP-TFII. Conversely, transactivation by COUP-TFII was repressed by increasing amounts of HNF-4alpha, indicating that COUP-TFII antagonizes repression of the *PED/PEA-15* gene by HNF-4alpha. Moreover, ChIP experiments in HepG2 cells demonstrate that HNF-4alpha binding to the PED/PEA-15 promoter is reduced by increasing amounts of COUP-TFII (Fig.13B), while the opposite was obtained in Hela cells in which COUP-TFII binding to the PED/PEA-15 promoter is antagonized by increasing amount of HNF-4alpha (Fig.13C).

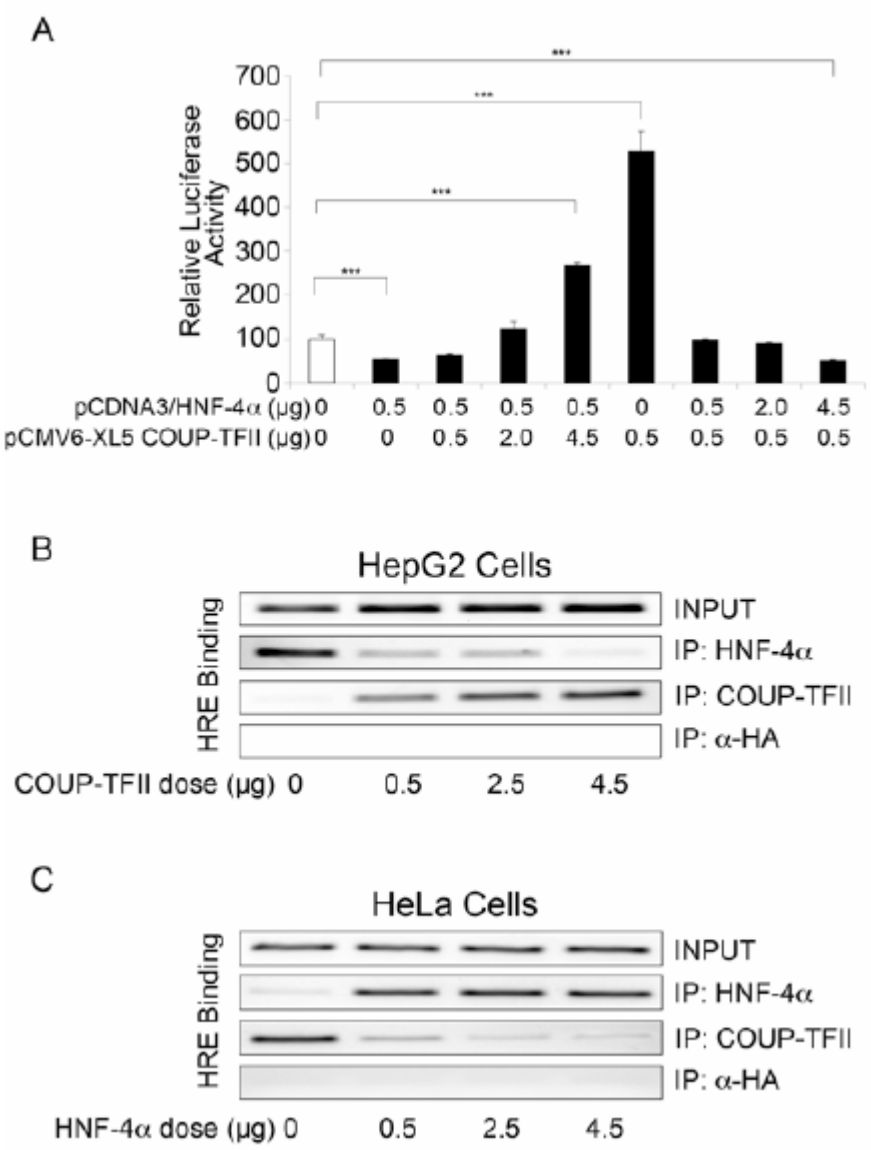

Figure 13 – Antagonistic effects of HNF-4alpha and COUP-TFII on PED/PEA-15 promoter. Hela cells were transiently transfected with 2 μg of the pPED477 *PED/PEA-15* promoter-luciferase construct alone (open bar) or in combination with the indicated amounts of the pCMV6-XL5COUP-TFII and the pCDNA3/HNF-4alpha plasmids. Luciferase activity was assayed and normalized as described under Materials and Methods. Bars represent the mean ± SD of three independent experiments. Asterisks denote statistically significant differences ($p < 0.001$). (B, C) ChIP assays. Soluble chromatin was prepared from HepG2 (B) and Hela (C) cell lines as described under Materials and Methods and immunoprecipitated with HNF-4alpha, COUP-TFII and HA antibodies. Total (INPUT) and immunoprecipitated DNAs were amplified using primer pairs covering HNF-4alpha RE on the PED/PEA-15 promoter. The photographs shown are representative of three independent experiments.

CHAPTER 4

HNF-4alpha directs histone deacetylation and methylation to silence Ped/Pea-15 expression in human cultured hepatocytes

RESULTS

HNF-4alpha expression results in nucleosome assembly at the promoter of *PED/PEA-15*

We have already shown that HNF-4alpha inhibits *PED/PEA-15* expression by binding the HRE and, at least in part, by competing for the binding with COUP-TFII.

By the way, the molecular mechanism by which HNF-4alpha exerts its action on *PED/PEA-15* expression is still unclear.

As an attempt to clarify this mechanism, we analysed the region immediately downstream the HRE on *PED/PEA-15* promoter by using the free available software RECON [94] which calculates the nucleosome formation potential of a given DNA sequence. We localized two potential nucleosomes on *PED/PEA-15* promoter, and we called them NUC81 (N1) and NUC208 (N2). N1 is closed to the HRE, whilst N2 overlaps the transcription start site of the gene (Fig.14A).

To further validate bioinformatic results, we examined protein occupancy at *PED/PEA-15* promoter both in wild-type Hela and HepG2 cells and in $Hela_{HNF}$ and $HepG2_{sh}$ cells as well.

As previously described, Hela and HepG2 cells significantly differ from each other in Ped/Pea-15 mRNA levels, with Hela cells having higher *PED/PEA-15* expression than HepG2 cells. Furthermore, in Hela cells HNF-4alpha is barely absent and not bound to the HRE differently from HepG2 cells which show high HNF-4alpha expression and binding. Hela cells were transfected with a full-length HNF-4alpha cDNA ($Hela_{HNF}$

cells) to silence *PED/PEA-15* expression whilst *PED/PEA-15* expression was restored in HepG2 cells, by silencing HNF-4alpha with a specific shRNA ($HepG2_{sh}$ cells).

To analyse protein occupancy at *PED/PEA-15* promoter we used a Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) assay. Once protein and DNA are crosslinked, chromatin is prepared and sheared by sonication. Then protein-free DNA is then purified by a phenol/chloroform extraction, recovered in the aqueous phase and subjected to PCR amplification with specific primers.

In these cell lines we performed FAIRE followed by end-point PCR using specific primers spanning the N1 (N1F/R) and N2 (N2 F/R) regions, as schematically depicted in Fig.14A. As shown in Fig.14B protein/DNA complexes from each cell type were sonicated at comparable levels (0.1 – 0.5 Kb) and, in these conditions, N1 and N2 regions were specifically occupied in HepG2 and $Hela_{HNF}$ cells (Fig. 14C), whilst these regions were free of proteins in Hela and $HepG2_{sh}$ cells.

A preparation of input DNA, obtained by decrosslinking protein/DNA complexes, is included as an internal control of total cellular DNA.

These data clearly show that HNF-4alpha binding to the HRE directs protein assembly at the promoter of *PED/PEA-15*.

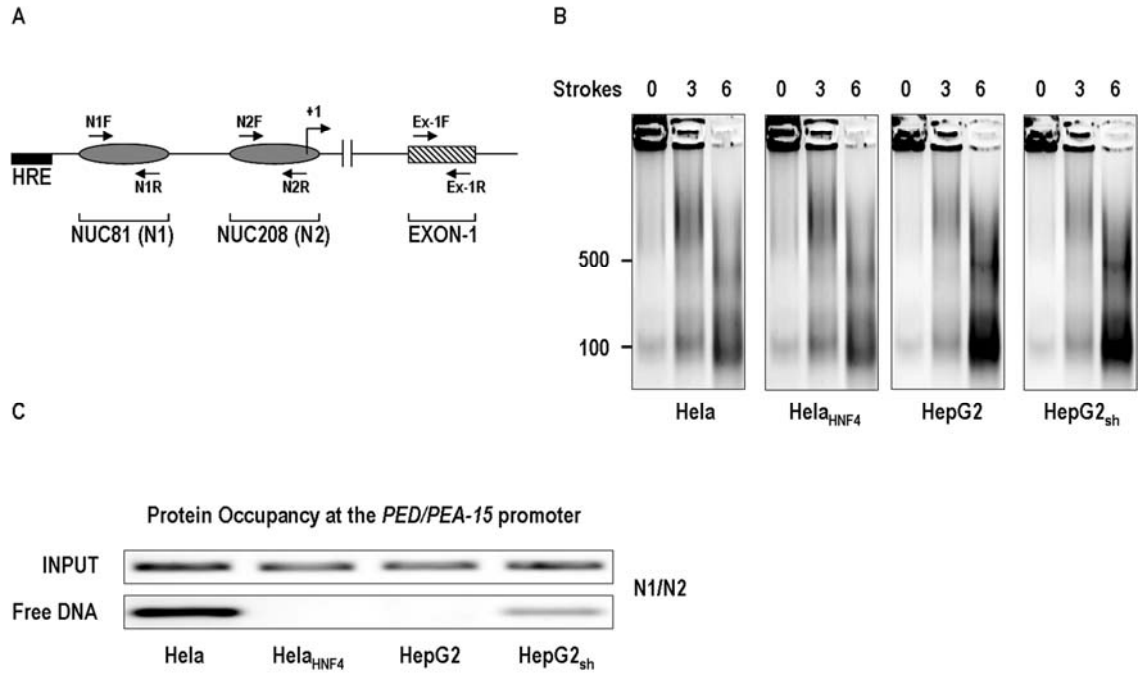

Figure 14 – Protein Occupancy at the promoter of Ped/Pea-15. (A) Schematic representation of nucleosome positioning at the promoter region of the Ped/Pea-15 gene. (B) Protein and DNA from each of the four cell types were crosslinked with 1% Formaldehyde and obtained chromatin was sheared by sonication. As shown in the picture, after 6 strokes of sonication chromatin was sheared at comparable levels in all cell types with DNA fragments ranging from 0.1 to 0.5 kilobases. (C) DNA recovered in the aqueous phase by phenol/chloroform extraction was subjected to end-point PCR with N1F/R and N2F/R primer pairs to analyse protein occupancy at these regions. As shown, only in Hela and HepG2$_{sh}$ cells, which have high Ped/Pea-15 expression levels and low HNF-4alpha binding, N1 and N2 regions are free of proteins, whilst in Hela$_{HNF}$ and HepG2 cells, having high HNF-4alpha binding and low Ped/Pea-15 expression levels, no PCR product is obtained for N1 and N2 regions indicating they are occupied by proteins. A preparation of input DNA, obtained by decrosslinking protein/DNA complexes, is included as an internal control of total cellular DNA.

To further confirm FAIRE results and assess the presence of nucleosomes on *PED/PEA-15* promoter, we performed MNase Protection Assay on chromatin digested up to mononucleosomes, as representatively shown by the picture in Fig.15A. Mononucleosomal DNA was then extracted from the agarose gel and subjected to end-point PCR with specific primers, so that PCR product was visible only for DNA regions protected from enzymatic digestion. As shown in figure 15B, both N1 and N2 regions are protected from MNase digestion in Hela$_{HNF}$ as well as in HepG2 cells where HNF-4alpha is bound to the HRE, whilst they are

sensitive to MNase in Hela and $HepG2_{sh}$ cells where HNF-4alpha is not bound to the HRE. These results strongly suggest that HNF-4alpha directs nucleosome assembly on *PED/PEA-15* promoter once bound to the HRE. A preparation of undigested chromatin is included as an internal control of DNA quality, whilst the Ex-1 region is used as a control of MNase digestion because it represents the exon-1 of *PED/PEA-15* gene which is never protected from the enzymatic activity as shown in Fig.15C.

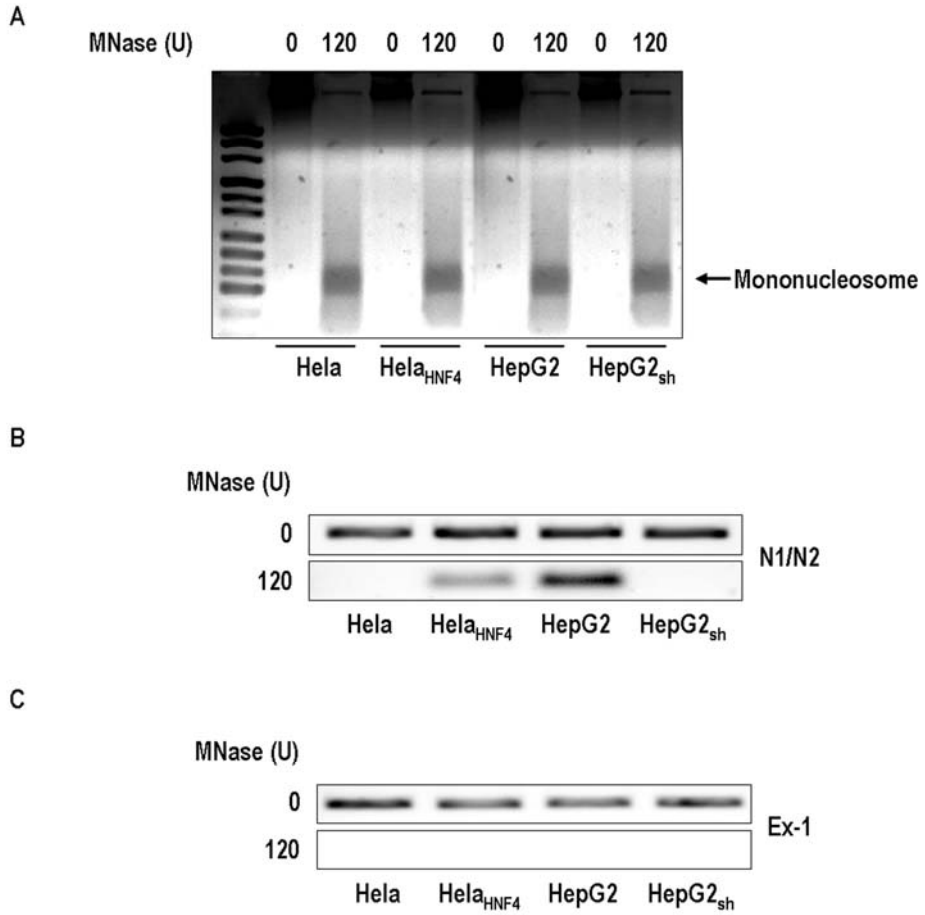

Figure 15 – HNF-4alpha dependent nucleosome assembly at the promoter of Ped/Pea-15. (A) Chromatin obtained from Hela, $Hela_{HNF}$, HepG2 and $HepG2_{sh}$ cells is digested with 120 U of MNase for 30' to obtain mononucleosomes. Once separated on an agarose gel, mononucleosomal DNA (indicated by the arrow in the picture) is purified and used as template for PCR reaction with specific oligos. (B) N1 and N2 regions are protected from MNase digestion in cells which have high levels of HNF-4alpha expression and binding to the HRE, as happens for $Hela_{HNF}$ and HeopG2 cells. By contrary, they are not protected in Hela and $HepG2_{sh}$ cells which have very low levels of HNF-4alpha expression and binding to the HRE. (C) Analysis of the exon-1 region is included as a control of MNase digestion.

HNF-4alpha expression results in histone deacetylation and methylation of the *PED/PEA-15* promoter

We next focused on the influence of HNF-4alpha on the epigenetic state of the nucleosomes on *PED/PEA-15* promoter. We analysed epigenetic marks characteristic of transcriptional activation and repression both in wild-type Hela and HepG2 cells and in $Hela_{HNF}$ and $HepG2_{sh}$ cells as well. Histone modifications were analysed by ChIP assays followed by Real-Time PCR for the N1 region and end-point PCR for the N2 region. Consistently with the reduced expression of *PED/PEA-15* gene in HepG2 and $Hela_{HNF}$ cells compared with Hela and $HepG2_{sh}$ cells respectively, general acetylation of histone H3 (AcH3) was decreased at both N1 and N2 (Fig.16A).

Similarly, there was a reduction, in the presence of HNF-4alpha, of di-methylated histone H3 at lysine 4 (H3K4), a mark usually associated with active genes [95], at both N1 and N2 regions (Fig.16B), and an enrichment in the di-methylation of histone H3 at lysine 9 (H3K9), a mark characteristic of repressed gene promoters [96]. In particular, H3K9, involved in transcriptional repression in euchromatic regions [97], was increased in HNF-4alpha expressing cells at both N1 and N2.

We conclude that HNF-4alpha expression and binding to the HRE results in a deacetylation of the histone H3 and in an enrichment of repressive histone modifications, leading to a reduction of *PED/PEA-15* gene expression.

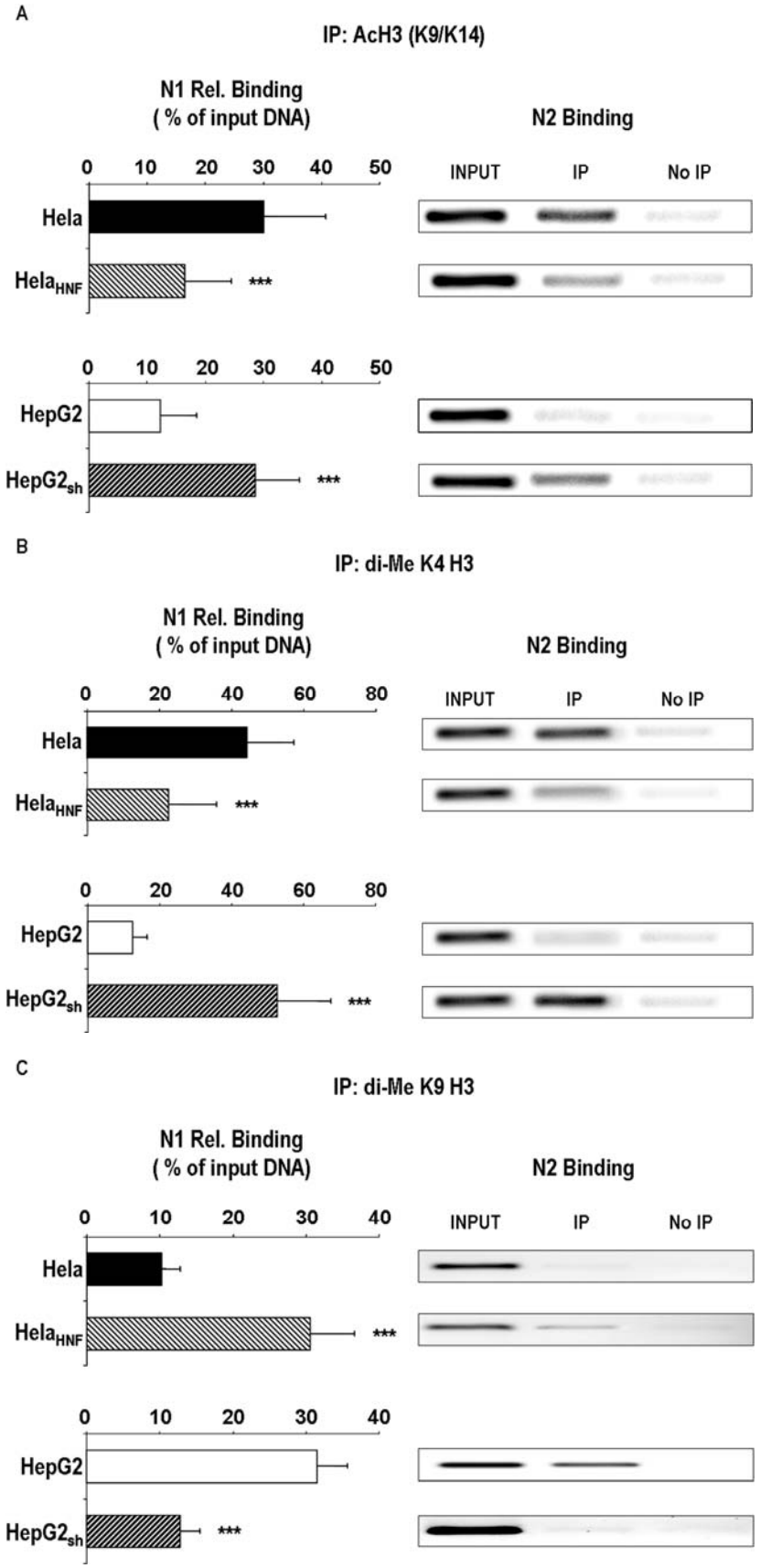

Figure 16 – HNF-4alpha dependent histone modifications at the promoter of the *PED/PEA-15* gene. Chromatin obtained from Hela, Hela$_{HNF}$, HepG2 and HepG2$_{sh}$ cells is sheared by sonication and subjected to immuno-precipitation with antibodies directed against the acetylated Lys9 and Lys14 on histone H3 (A), the di-methylated

Lys4 on histone H3 (B) and the di-methylated Lys9 on histone H3 (C). The DNA eluted and purified from the immuno-complexes is then analyzed by Real-Time PCR or end-point PCR. A preparation of input DNA, obtained by decrosslinking protein/DNA complexes from non immunoprecipitated lysate, is included as an internal control of total cellular DNA, and a NoIP sample is included as a negative control. Expression of HNF-4alpha and its binding to the HRE reduces markers of active expression on *PED/PEA-15* promoter and enriches markers of inactive chromatin, thus leading to a reduction in Ped/Pea-15 expression levels.
*** stands for $p < 0.001$

Restoration of PED/PEA-15 expression in HepG2 cells by treatment with 5-Aza-dC and TSA

To examine whether *PED/PEA-15* silencing in HepG2 cells could be restored, we treated HepG2 cells for 24h with 10μM of the DNA methylation inhibitor 5-deoxy-Aza-Cytidine (5-Aza-dC) [98], or 330nM of the histone deacetylase inhibitor, Trichostatin A (TSA), [99]. The two drugs were also used in combination to evaluate a potential synergism between their different mechanisms of action.

After treatment, cells were harvested and analysed for HNF-4alpha and *PED/PEA-15* expression compared with untreated cells. Real-Time RT-PCR revealed an increase in HNF-4alpha and *PED/PEA-15* expression upon 5-Aza-dC and TSA treatment compared with untreated cells (Fig.17A and B), indicating that DNA methylation and histone deacetylation are both involved in the control of their expression.

We next examined the possibility that the demethylation and the hyperacetylation of *PED/PEA-15* promoter following 5-Aza-dC and TSA treatment may affect HNF-4alpha binding thus leading to an increase in *PED/PEA-15* expression. As shown in Fig.17C, HNF-4alpha binding to the HRE on *PED/PEA-15* promoter was unaffected (or slightly increased) after 5-Aza-dC and TSA treatment consistently with the increasing expression.

To decipher whether DNA methylation and epigenetic modifications in *PED/PEA-15* promoter are related, we examined histone H3 acetylation and methylation upon treatments, by ChIP assay. As shown in Fig.17D,

histone H3 was much more acetylated on both N1 and N2 upon treatment with TSA, alone or in combination with 5-Aza-dC, compared with untreated cells. No significant differences were observed with 5-Aza-dC alone. Consistently, the lysine 9 on histone H3 of both N1 and N2 was less methylated in HepG2 cells treated with TSA, alone or in combination with 5-Aza-dC, compared with untreated cells. Once again no differences were observed with 5-Aza-dC alone.

These data strongly suggest that DNA methylation and histone modifications are two independent mechanisms potentially involved in the regulation of *PED/PEA-15* expression in HepG2 cells. It is even clear that HNF-4alpha functions as a scaffold protein to recruit a corepressor with histone deacetylase activity to silence *PED/PEA-15* expression in HepG2 cells.

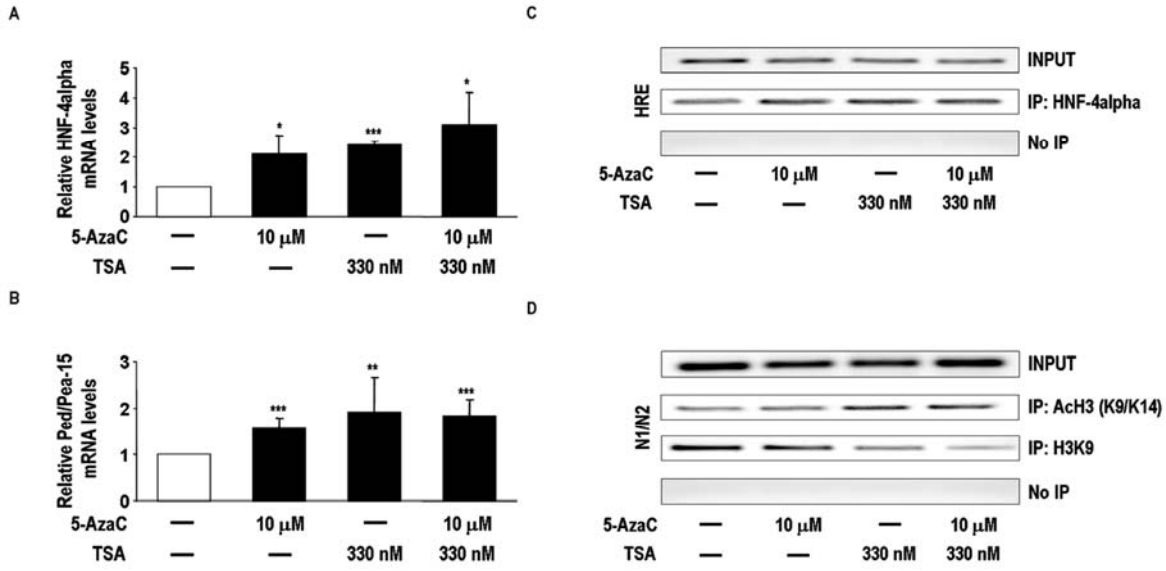

Figure 17 – 5-Aza-dC and TSA treatment restores Ped/Pea-15 expression in HepG2 cells. HepG2 cells were treated for 24h with 10μM of 5-Aza-dC and/or 330nM of TSA to try to restore *PED/PEA-15* expression. After treatment, cells were harvested to evaluate both HNF-4alpha expression and binding to the HRE (A,C) and *PED/PEA-15* expression and histone modifications of its promoter in terms of general acetylation of histone H3 and di-methylation of the Lys9 of histone H3 (B,D). As shown in the figure, treatments increase both HNF-4alpha and *PED/PEA-15* expression. In particular, TSA treatment has a strong effect on H3 acetylation and H3 Lys9 di-methylation of the *PED/PEA-15* promoter, indicating that HNF-4alpha acts by recruiting a corepressor complex with histone deacetylase (HDAC) activity.
*** stands for $p < 0.001$.

HNF-4alpha targets repressive enzymes to the PED/PEA-15 promoter leading to its tight packaging and inhibition of transcription

Given that HNF-4alpha expression and binding to the HRE on *PED/PEA-15* promoter leads to a decrease in histone acetylation and to a subsequent increase in the di-methylation of the Lys9 on histone H3, we next investigated the binding of histone-modifying enzymes to the *PED/PEA-15* promoter, in the presence and absence of HNF-4alpha.

Recent data in literature show that HNF-4alpha interacts with the Silencer Mediator of Retinoic acid and Thyroid hormone receptor (SMRT) [100].

SMRT is a corepressor complex with HDAC activity, which is recruited by hormone-free receptors, such as thyroid hormone and retinoic acid receptors, and mediates histone deacetylation to silence the expression of target genes.

Using ChIP assay, we demonstrated an increase in the binding of the SMRT corepressor complex to the HRE in HepG2 cells, compared to Hela cells (Fig.18A). Importantly, recruitment of this repressive enzyme was entirely dependent on the presence of HNF-4alpha whilst its expression was not affected by the expression of HNF-4alpha, as shown in figure 18B where relative mRNA levels of SMRT are comparable in Hela and HepG2 cells, differently from HNF-4alpha, which is clearly much more expressed in HepG2 rather than in Hela cells (Fig.18B).

These data clearly indicate that SMRT recruitment was strictly dependent on HNF-4alpha and was not a reflection of its relative expression levels.

To further assess the role of HNF-4alpha in the recruitment of SMRT to the promoter of Ped/Pea-15, we evaluated the assembly of HNF-4alpha/SMRT complex on the HRE by ReChIP assay in our cellular models. Briefly, sheared chromatin from each cell type was first immunoprecipitated with the HNF-4alpha antibody and the immuno-complex was then subsequently immunoprecipitated with the SMRT

antibody. The DNA purified from the immuno-complex was amplified with the primers for the HRE. As you can see from the figure 18C the formation of the HNF-4alpha/SMRT complex is dependent on HNF-4alpha binding, because it is increased both in $Hela_{HNF}$ and HepG2 cells when compared with Hela and $HepG2_{sh}$ cells respectively. We further demonstrated the interaction between HNF-4alpha and SMRT by co-immunoprecipitation followed by western blot assay. Briefly, nuclear extracts from each cell type were immunoprecipitated with the SMRT antibody and then the immunocomplexes blotted with the HNF-4alpha antibody. Once again, the amount of SMRT, being comparable in all cell lines, is not dependent upon HNF-4alpha expression whilst the interaction between them is completely dependent on HNF-4alpha expression because this interaction is much stronger in $Hela_{HNF}$ and in HepG2 cells compared with Hela and $HepG2_{sh}$ cells respectively, as shown in figure 18D.

Our data clearly demonstrate that HNF-4alpha interacts with and recruits SMRT to *PED/PEA-15* promoter and this can lead to the silencing of Ped/Pea-15 expression via deacetylation and packaging of the core-promoter of the gene.

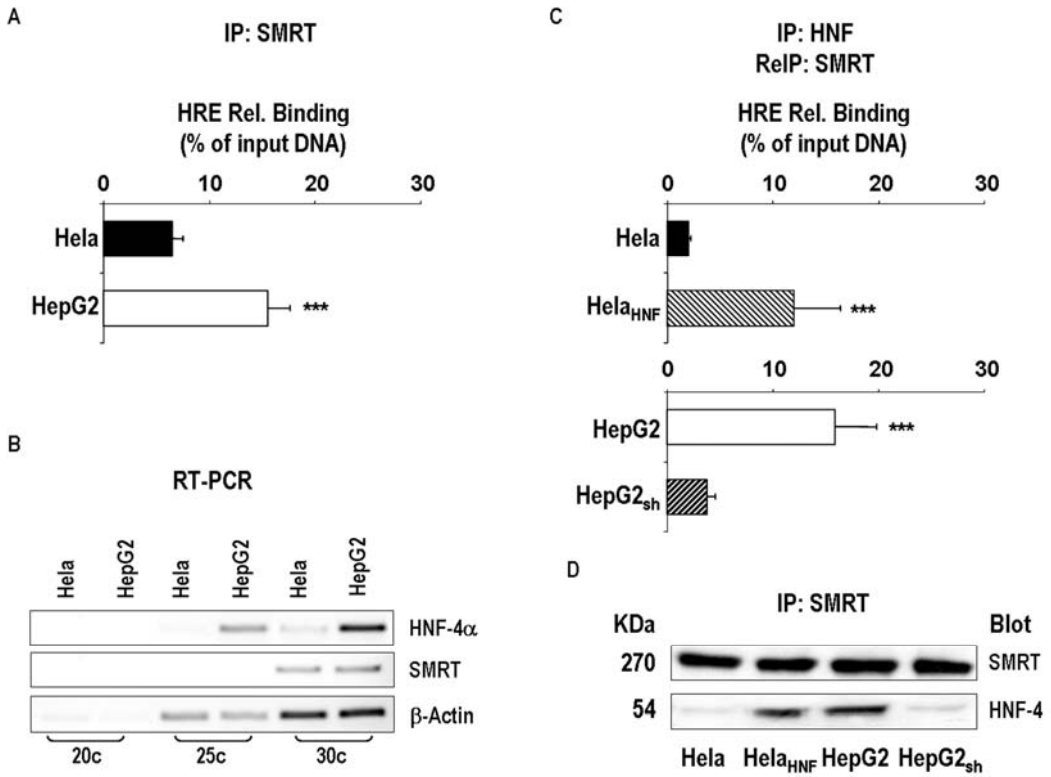

Figure 18 – HNF-4alpha interacts with and recruits SMRT to Ped/Pea-15 promoter. (A) Chromatin from Hela and HepG2 cells was immunoprecipitated with the SMRT antibody and eluted DNA amplified with the primers for the HRE. As shown, the binding of SMRT is much more stronger in HepG2 cells which have high levels of endogenous HNF-4alpha. Rather than the recruitment to the HRE, SMRT expression is not dependent on HNF-4alpha expression. (B) 1 μg of total cellular RNA from Hela and HepG2 cells was reverse transcribed to obtain cDNA and 12.5 ng of cDNA was used in the RT-PCR with HNF-4alpha and SMRT specific primers. As shown in the picture, Hela and HepG2 cells have comparable levels of SMRT mRNA even though HepG2 cells have higher HNF-4alpha levels. (C) Sheared chromatin from Hela, Hela$_{HNF}$, HepG2 and HepG2$_{sh}$ cells was sequentially immunoprecipitated with HNF-4alpha and SMRT antibodies. Ped/Pea-15 promoter occupancy was then quantified by Real-Time PCR with primers for the HRE and after normalization over input DNA, used as a control of total cellular DNA. As shown in the figure, the formation of the HNF-4alpha/SMRT complex is dependent on HNF-4alpha expression and binding. (D) Nuclear extracts from Hela, Hela$_{HNF}$, HepG2 and HepG2$_{sh}$ cells were immunoprecipitated with the SMRT antibody and the immunocomplexes separated on a SDS-PAGE and blotted with HNF-4alpha antibody, showing a much strong interaction between HNF-4alpha and SMRT in Hela$_{HNF}$ and HepG2 cells which have high levels of HNF-4alpha expression and binding.
*** stands for $p < 0.001$

Histone deacetylation has been shown to induce transcriptional repression of genes at euchromatic regions [73] by mediating the tight packaging of core-promoters.

To go further insight this mechanism in the case of *PED/PEA-15*, we evaluated the packaging of the *PED/PEA-15* promoter by ReChIP experiments using H3K9 antibody in the first ChIP and HNF-4alpha or SMRT antibodies in the second ChIP. As shown in Fig. 19, we were able to detect HNF-4alpha and SMRT together with H3K9 at both N1 and N2 regions in $Hela_{HNF}$ and HepG2 cells compared with Hela and $HepG2_{sh}$ cells, where signals were barely undetectable. These evidences clearly indicate a HNF-4alpha-dependent packaging of the *PED/PEA-15* promoter.

Taken together, our results lead us to conclude that HNF-4alpha selectively targets SMRT to the *PED/PEA-15* promoter resulting in its tight packaging and repression of gene expression.

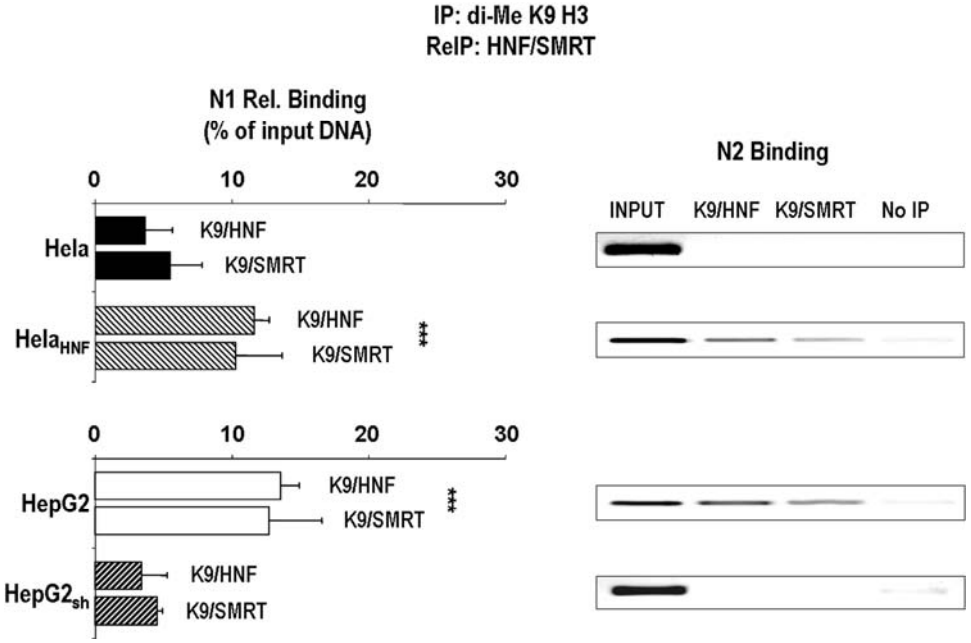

Figure 19 – HNF-4alpha mediates the packaging of the *PED/PEA-15* promoter. To evaluate the HNF-4alpha-dependent packaging of the *PED/PEA-15* promoter, sheared chromatin from Hela, $Hela_{HNF}$, HepG2 and $HepG2_{sh}$ cells was sequentially immunoprecipitated with the H3K9 and the HNF-4alpha or the SMRT antibodies and eluted DNA amplified with primers N1F/R and N2F/R. As shown in the figure, both at the N1 and N2 regions, HNF-4alpha functions as a scaffold protein to recruit SMRT and induce the tight packaging of the *PED/PEA-15* promoter. All the effect observed are completely dependent upon HNF-4alpha expression and binding to the HRE. *** stands for $p < 0.001$

Inhibition of SMRT expression restores Ped/Pea-15 expression reverting the inhibitory effect of HNF-4alpha

As already demonstrated in this work that the SMRT corepressor interacts with HNF-4alpha on both HRE and nucleosomes of the Ped/Pea-15 promoter.

To assess the contribution of SMRT to the HNF-4alpha dependent repression of *PED/PEA-15* expression, HepG2 cells were transfected with a SMRT selective siRNA (panS), or a non-specific control (Con.) as previously reported by Peterson T.J. et al. [102].

SMRT and *PED/PEA-15* expression were measured as both mRNA and protein levels (Fig.20A and 20B) showing that selective depletion of SMRT significantly increased *PED/PEA-15* expression. ChIP experiments from both panS and Con. transfected cells show that HNF-4alpha binding to the HRE was not affected by panS, whilst SMRT binding to the HRE as well as HNF-4alpha/SMRT interaction on *PED/PEA-15* promoter was significantly reduced (Fig.20C). Furthermore we analyzed histone modifications at both N1 and N2 regions in panS and Con. transfected cells. As shown in Fig.20D, the general acetylation of histone H3 was strongly increased at both N1 and N2 regions, as expected after SMRT silencing, and, as a consequence of increased H3 acetylation, H3K9 levels were significantly reduced.

These data clearly indicate that SMRT plays a critical role in the HNF-4alpha dependent inhibition of *PED/PEA-15* expression as well as in the HNF-4alpha-dependent histone modifications at *PED/PEA-15* promoter.

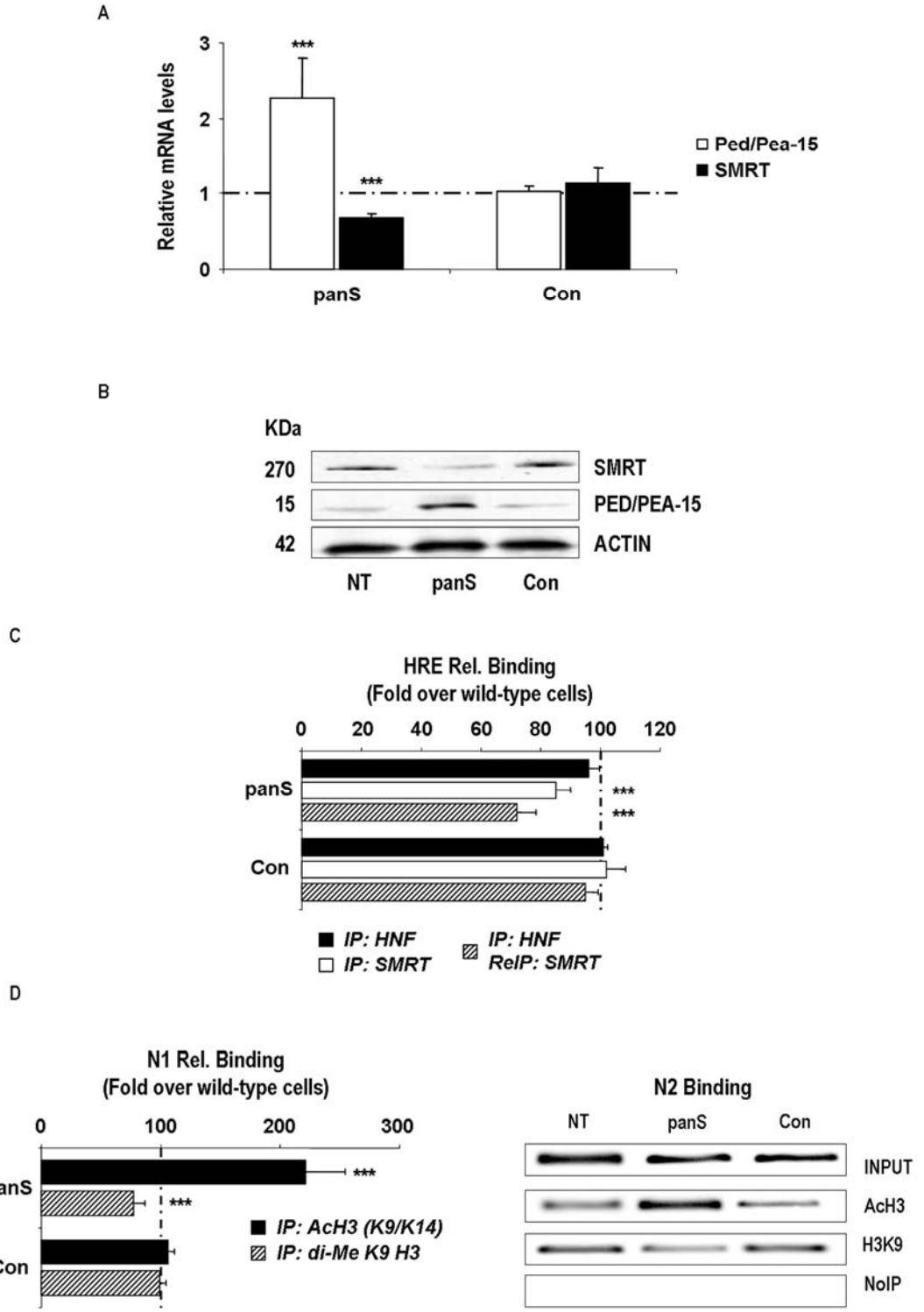

Figure 20 – SMRT silencing in HepG2 cells reverts the inhibitory effect of HNF-4alpha on *PED/PEA-15* expression. SMRT expression was silenced in HepG2 cells with a specific siRNA (panS) whilst a scrambled oligonucleotide (Con.) was used as a negative control. (A, B) SMRT and *PED/PEA-15* mRNA and protein levels were evaluated after transfection showing a significant decrease in SMRT levels and a parallel increase in *PED/PEA-15* levels as both mRNA and protein. (C) HNF-4alpha and SMRT binding to the HRE as well as the interaction between HNF-4alpha and SMRT on *PED/PEA-15* promoter were evaluated after transfection by ChIP and ReChIP assays. As shown, whilst HNF-4alpha binding is not affected by SMRT silencing, SMRT binding as well as HNF-4alpha and SMRT interaction are

significantly reduced. (D) Consistently with the reduced formation of HNF-4alpha/SMRT complex on *PED/PEA-15* promoter, general acetylation of histone H3 was significantly increased at both N1 and N2 regions and, as expected, the di-methylated Lys9 on histone H3 was reduced. *** stands for $p < 0.001$

Chapter 5

Discussion

Several lines of evidence indicate that a multitude of concurrent alterations contribute to type 2 diabetes onset and progression [102-104]. Amongst these defects, the *PED/PEA-15* gene has been found to be overexpressed in about 30% of individuals affected by type 2 diabetes and in their first degree relatives. Studies in cellular and animal models have shown a cause-effect relationship between the overexpression of *PED/PEA-15* and impaired insulin action [7; 8; 19-21]. Thus, clarifying the mechanism of *PED/PEA-15* transcriptional regulation will help understanding its abnormalities in type 2 diabetes. In the present study we have isolated and further characterized the *PED/PEA-15* promoter region and we have identified cis-elements involved in the regulation of *PED/PEA-15* human gene expression.

The transcription start site of the *PED/PEA-15* gene has already been mapped [105]. Analysis of the 5' genomic sequence revealed that this gene lacks a TATA box and the first 600 bp flanking the transcription start region have a high GC content (64%), including several consensus Sp1 sites. These data are consistent with our results showing that the shortest 5'-flanking fragment spanning nucleotides -39/+58 contains all the elements necessary to achieve basal promoter activity.

Deletion analyses showed that HNF-4alpha, a member of the steroid receptor class of transcription factors, binds to the -419/-309 fragment and represses *PED/PEA-15* transcription through this region (Fig. 7, 8). Indeed transfection experiments of both an HNF-4alpha expression vector in Hela cells and an HNF-4alpha specific shRNA clone in HepG2 cells confirmed that this transcription factor is capable to repress *PED/PEA-15* expression both at the RNA and at the protein levels (Fig. 9 and 11).

EMSA and ChIP assays have further shown that the promoter site recognized by HNF-4alpha is located between nt -335 and -320 (Fig. 10). This element closely resembles the HNF-4alpha REs found in other genes and the GGGGCA A AGGTCA consensus HNF-4alpha binding site [106]. In addition we have shown that the same responsive element is recognized by COUP-TFII, another member of the orphan receptor family (Fig. 12). We found that COUP-TFII activates *PED/PEA-15* gene expression by binding to the HRE (Fig. 12), while HNF-4alpha functions as a transcriptional repressor. The observation that these two transcription factors bind to the HRE in a mutually exclusive manner (Fig. 13) suggests that their mechanism of action involves direct competition at the level of DNA binding. Thus, transcription of the *PED/PEA-15* gene is dependent upon the intracellular balance of these positive and negative regulatory factors. Competition for DNA binding has been described for other members of the steroid receptor superfamily which because of their highly related zinc finger DNA binding domains, can interact with overlapping or identical DNA elements. For example, estrogen and thyroid hormone receptors exert opposite regulatory effects via their competitive binding to the estrogen response element [107].

In certain cell types, where COUP-TFII occupies the HRE, transcription of *PED/PEA-15* gene is enhanced while in other cell types, such as the hepatocytes, where HNF-4alpha is present in relatively abundant amounts, COUP-TFII is replaced by HNF-4alpha and *PED/PEA-15* transcription is repressed. At variance from the *PED/PEA-15* gene, COUP-TFII most often serves as a transcriptional silencer [50] while HNF-4alpha is an activator [44]. There is also evidence, however, indicating that the function of many nuclear hormone receptors is dependent upon the actions of distinct receptor binding cofactors that differentially recognize occupied and unoccupied receptors [51-58].

Indeed, nuclear receptors often interact with co-regulators affecting their target genes in a tissue- and gene-specific manner [108]. For instance, it has been reported that PGC-1alpha co-activates HNF-4alpha and induces CYP7A1 gene transcription during starvation in mice [109]. But Song et al. also demonstrated that Prox1, an early specific marker for liver and pancreas development from the foregut endoderm, interacts with HNF-4alpha and suppresses CYP7A1 gene transcription [110]. These authors have generated evidence suggesting that Prox1 competes with PGC-1alpha for HNF-4alpha binding and thus inhibits PGC-1alpha co-activating activity. The identification of ligands cooperating with HNF-4alpha and COUP-TFII in the regulation of *PED/PEA-15* gene is currently in progress in the laboratory and expected to help clarifying the mechanism linking *PED/PEA-15* overexpression and the genes controlling its expression. Indeed, *PED/PEA-15* overexpression likely represents a molecular abnormality downstream distinct diabetes risk genes and serves as an effector of these genes.

In conclusion, in this first part of my work, I have cloned and characterized the PED/PEA-15 promoter, and I have further shown that two transcription factors, HNF-4alpha and COUP-TFII, compete for binding to the HRE site on PED/PEA-15 gene promoter. While COUP-TFII increases PED/PEA-15 gene expression, HNF-4alpha antagonizes PED/PEA-15 transactivation.

In the previous section of my work, I have clearly established the importance of the orphan nuclear receptor HNF-4alpha in the regulation of the *PED/PEA-15* gene expression. In particular, I have shown that HNF-4alpha inhibits *PED/PEA-15* gene expression by binding its response element (HRE) on the promoter of the gene and by competing for the binding with the transcriptional activator COUP-TFII.

In this section, I demonstrate that HNF-4alpha inhibits *PED/PEA-15* expression by inducing an eterochromatinization of the core-promoter of the gene. In particular, it mediates the assembly of nucleosomes (Fig. 15) and leads to an enrichment in markers typical of silent chromatin on *PED/PEA-15* promoter, such as deacetylated lysines 9 and 14 on histone H3 and subsequent di-methylated lysine 9 with a parallel decrease in di-methylated lysine 4 (Fig. 16).

Nucleosome, which consists of 146 bp of DNA wrapped around an octamer of core histone proteins (H2A, H2B, H3 and H4), is the fundamental repeating unit of chromatin. Linker histones of the H1 class associate with DNA between single nucleosomes establishing a higher level of organization, the so-called “solenoid” helical fibers (30 nm fibers). Core histone proteins are evolutionarily conserved and consist mainly of flexible amino-terminal tails protruding outward from the nucleosome, and globular carboxy-terminal domains making up the nucleosome scaffold. Histones function as acceptors for a variety of post-translational modifications, including acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues [66; 111-114].

One major challenge in chromatin biology is connecting particular modifications with distinct biological functions and vice versa. One of the better-understood histone modifications in that aspect is histone acetylation. It is now generally accepted that hyperacetylated histones are mostly associated with activated genomic regions, at both local and global levels. By contrast, deacetylation (leading to hypoacetylation) mainly results in repression and silencing [74; 115-118].

Interestingly, histone methylation appears to have multiple effects on chromatin function in a system and site-specific manner. Methylation of

H3 on K9, for example, is largely associated with silencing and repression in many species. Methylation of H3 on K4, on the other hand, is often associated with active or permissive chromatin regions.

I further demonstrates that HNF-4alpha expression in Hela cells (which endogenously express low HNF-4alpha levels) leads to a decrease in histone H3 acetylation in the promoter of the *PED/PEA-15* gene and, conversely, HNF-4alpha silencing in HepG2 cells (which endogenously express high HNF-4alpha levels) leads to an increase in this marker typical of active promoters (Fig. 16). However, trichostatin A treatment of HepG2 cells completely reverts HNF-4alpha action on *PED/PEA-15* gene expression and histone modifications at *PED/PEA-15* promoter (Fig. 17).

Thus, it appears that HNF-4alpha provides a scaffold for the assembly of histone deacetylating enzymes, thereby providing a mechanistic link between the silencing of *PED/PEA-15* expression in HepG2 cells and the binding of HNF-4alpha to its promoter.

Repression of gene expression by nuclear receptors is mediated by limiting cellular proteins that are recruited to the C-terminal ligand binding domain (LBD) of the receptor [119]. These proteins, termed corepressors, must fulfill four important criteria, namely: (1) interaction with the unliganded receptor; (2) dissociation upon receptor binding of activating ligand; (3) potentiation of receptor repression; and (4) intrinsic ability to repress transcription of genes to which they are recruited.

SMRT and N-CoR are related corepressors that influence the transcriptional activity of many members of the nuclear receptor superfamily. Current working models suggest that in the presence of an hormone free nuclear receptor, SMRT and N-CoR and their associated inhibitory molecules are recruited to the receptor and block its activation of receptor-dependent gene expression.

These large molecules can be divided into a C-terminal region that interacts with nuclear receptors and an N-terminal region that transmits the repression signal to the basal machinery and to chromatin.

Here we show that HNF-4alpha recruits SMRT to the promoter of the Ped/Pea-15 gene (Fig. 18) leading to a decrease in the acetylation of histone H3 mainly on lysines 9 and 14 (Fig. 19). Conversely, the depletion of SMRT with a specific siRNA completely prevents the inhibitory effects of HNF-4alpha on Ped/Pea-15 expression and reverts the HNF-4alpha-induced chromatin modifications (Fig. 20).

An ever growing number of modification sites on both histone-tail and non-tail domains have been identified [66]. For example, lysine residues in histones can be modified by acetylation, mono-ubiquitination or mono-, di-, and tri-methylation. [66; 120]. Although it remains unclear as to what extent, if at all, individual residues undergo “choices” of modification, it is well documented that H3-K9 and H3-K14 can be either acetylated or (mono, di-, tri-) methylated [121; 122]. Obvioulsy different marks on the same site cannot co-exist, and therefore, they exclude each other. An acetyl group, for example, must be removed before a methyl group can be added and complexes that contain both histone deacetylases (HDACs) and HMTs have now been identified [123-125]. So far, the amino-terminal tail of histone H3 has the highest density of post-translational modifications mapped among all histones, and a complex pattern of putative combinations of marks is emerging. Methylation of H3-K9, for example, appears to trigger sequencial events leading ultimately to transcriptional repression [126]. At least in vitro, this mark can inhibit acetylation of the H3 tail (on K14, K18 and K23) by histone acetyltransferase (HAT) such as p300 [126], and methylation of H3-K4 by HMTs such as Set7 [126]. By contrast, H3-K4 methylation inhibits K9

methylation by Su(var)3-9, but promotes acetylation of H3 by p300 [126].

Finally my results indicate that HNF-4alpha induces the packaging of the PED/PEA-15 promoter acting as a scaffold for a "bridge-like" structure where SMRT, recruited by HNF-4alpha to PED/PEA-15 promoter, deacetylates histone H3, leads to the inhibitory di-methylation of H3-K9 and this results in the silencing of PED/PEA-15 expression.

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LIST OF ORIGINAL PUBLICATIONS

1) Ungaro P.*, **Teperino R.***, Mirra P., Cassese A., Fiory F., Perruolo G., Miele C., Laakso M., Formisano P. and Beguinot F.: *Molecular cloning and characterization of the human Ped/Pea-15 promoter reveals antagonistic regulation by HNF-4alpha and COUP-TFII* **J. Biol. Chem.** 2008 Sep.2 [epub ahead of print]
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2) Viparelli F., Paturzo F., Doti N., Cassese A., Marasco D., Dathan N.A., Monti S.M., Ungaro P., Sabatella M., Miele C., **Teperino R.**, Consiglio E., Pedone C., Beguinot F., Formisano P., and Ruvo M.: *Targeting of Ped/Pea-15 molecular interaction with phospholipase D1 enhances insulin sensitivity in skeletal muscle cells* – **J. Biol. Chem.** 2008 Jun 9

3) Miele C, Paturzo F, **Teperino R**, Sakane F, Fiory F, Oriente F, Ungaro P, Valentino R, Beguinot F, Formisano P.: *Glucose regulates diacylglycerol intracellular levels and protein kinase c activity by modulating diacylglycerol-kinase subcellular localization.* – **J. Biol. Chem.** 2007 Nov 2;282(44):31835-43

4) Perfetti A, Oriente F, Iovino S, Alberobello AT, Barbagallo AP, Esposito I, Fiory F, **Teperino R**, Ungaro P, Miele C, Formisano P, Beguinot F. : *Phorbol esters induce intracellular accumulation of the anti-apoptotic protein PED/PEA-15 by preventing ubiquitinylation and proteasomal degradation.* – **J. Biol. Chem.** 2007 Mar 23; 282 (12) 8648-57

5) Milano A., Gesualdi N.M., **Teperino R.**, Esposito F., Cocozza S. and Ungaro P.: *Oxidative DNA damage and activation of c-Jun N-terminal kinase pathway in fibroblasts from patients with hereditary spastic paraplegia* – **Cell. Mol. Neurobiol.**, Vol.5 n°8 – December 2005

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