Localization of hepatocyte nuclear factor-4α in the nucleolus and nucleus is regulated by its C-terminus

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ABSTRACT

Aims/Introduction: Mutations in hepatocyte nuclear factor-4α (HNF4α) lead to various diseases, among which C-terminal deletions of HNF4α are exclusively responsible for maturity onset diabetes of the young 1 (MODY1). MODY is an autosomal dominant disease characterized by a primary defect in insulin response to glucose, suggesting that the C-terminus of HNF4α is important for pancreatic β-cell function. To clarify the role of the C-terminus of HNF4α, changes in cellular localization and the binding ability to its regulator were examined, specifically in the region containing Q268, which deletion causes MODY1.

Materials and Methods: Cellular localization of mutant HNF4α were examined in monkey kidney 7 (COS7), Chinese hamster ovary, rat insulinoma and mouse insulinoma cells, and their binding activity to other proteins were examined by fluorescence resonance energy transfer (FRET) in COS7 cells.

Results: Although wild-type HNF4α was localized in the nucleoplasm in transfected cultured cells, Q268X-HNF4α was located predominantly in the nucleolus. Deletion analysis of the C-terminus of HNF4α showed that the S337X-HNF4α mutant, and other mutants with shorter amino acid sequences (S337-K194), were mostly localized in the nucleolus. HNF4α mutants with amino acid sequences shorter than the W192X-HNF4α mutant gradually spread to the nucleoplasm in accordance with their lengths. The A250X-HNF4α mutant was capable of causing the accumulation of HNF4α or the small heterodimer partner (SHP), one of the HNF4α regulators, in the nucleolus. However, the R154X-HNF4α mutant did not have binding ability to wild-type HNF4α or SHP, and thus was seen in the nucleus.

Conclusions: The C-terminus sites might play a key role in facilitating the nucleolar and subnucleolar localization of HNF4α.
(J Diabetes Invest, doi: 10.1111/j.2040-1124.2012.00210.x, 2012)

KEY WORDS: Fluorescence resonance energy transfer, Hepatocyte nuclear factor-4α, Nucleolus

INTRODUCTION

Maturity onset diabetes of the young 1 (MODY1) is an autosomal dominant disease that develops as a result of hepatocyte nuclear factor-4α (HNF4α) heterozygous mutations[1,2] and is characterized by a primary defect in insulin response to glucose[3,4]. A number of heterozygous HNF4α mutations causing MODY1 have been identified, and some of which are C-terminal truncated mutants, such as Q268X and R154X. Although mutations of HNF4α have been reported in various diseases, such as hemophilia, ovarian mucinous tumor and renal cell carcinoma[5–8], deletion mutations in the C-terminus are exclusively associated with MODY1[9,10]. HNF4α is a nuclear receptor that is synthesized in the cytoplasm and translocated into the nucleus, where it typically resides. HNF4α functions as a dimer[11]. It has been reported that Q268X shows no deoxyribonucleic acid (DNA)-binding activity in a reporter assay[12], whereas R154X retains DNA binding activity *in vitro*[13]. Q268X has also been shown to bind to wild-type (WT)-HNF4α in biochemical assays *in vitro*[12,14]. Because the nuclear distribution patterns of Q268X and WT differ, they are not considered to interact in living cells[14]. There is no report on the cellular localization of the Q268X-HNF4α and the Q268X-HNF4α/WT-HNF4α heterodimer to date. We hypothesized that elucidating the mechanisms that contribute to HNF4α intracellular localization could help us to understand how this molecule functions normally or in its mutated state. In the present study, we show that the Q268X-HNF4α mutant protein is localized primarily in the nucleoli of transfected Chinese hamster ovary (CHO) and monkey kidney 7 (COS7) cell lines, which do not express endogenous HNF4α. The Q268X-HNF4α mutant was also found in the nuclei of

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rat insulinoma (INS-1) and mouse insulinoma (MIN6) cell lines, which express normal endogenous HNF4α[15]. Systematic deletion analysis of the C-terminus region showed the specific sites that affect the dynamic nuclear localization of HNF4α. Changes in cellular localization can attenuate the ability for binding with the regulators of HNF4α. Among the regulators of HNF4α, the small heterodimer partner (SHP) is an effective inhibitor of HNF4α and its mutation causes early onset mild obesity with hyperinsulinemia[16]. HNF4α has been reported to inhibit the actions of the retinoid apoptosis inducer, 6-(3-[1-adamantyl]-4-hydroxyphenyl)-2-naphthalene carboxylic acid, by competitively binding to SHP and suppressing apoptosis[17]. Mutations in HNF4α are likely to alter its interactions with regulatory factors. We have previously shown that SHP translocates to the nucleus in association with HNF4α[18]. Therefore, we also examined the binding ability of SHP to C-terminal mutants of HNF4α. These results suggest an important role of the C-terminus region in the regulation of HNF4α function, implying that mutations in the specific sites might affect the pathogenesis of MODY.

MATERIALS AND METHODS
Construction of Expression Vectors
Among the HNF4α variants, HNF4α2 was used for its high transcriptional activation. Expression constructs used in the present study are complementary (c)DNA clones of the WT human HNF4α2 gene, and R127W and Q268X mutation genes that were isolated from white blood cells of a non-diabetic subject and a MODY1 patient[1,2], respectively. The genes were subcloned and ligated into enhanced green fluorescent protein (EGFP)-N3, enhanced yellow fluorescent protein (EYFP) or enhanced cyan fluorescent protein (ECFP). A FLAG epitope (MDYKDDDDKG) was introduced at the 5′ end for the construction of F-HNF4α, and F-HNF4α-CFP and F-HNF4α-YFP using P Blue script KS+ cytomegarovirus vector (pBK-CMV; Agilent Technologies Inc., Santa Clara, CA, USA). Deletion mutants of HNF4α (truncated from the C-terminus at various lengths) and single point mutants were constructed and ligated to ECFP or EYFP. SHP was isolated from the human heart cDNA library, amplified by polymerase chain reaction and tagged with GFP or FLAG epitope.
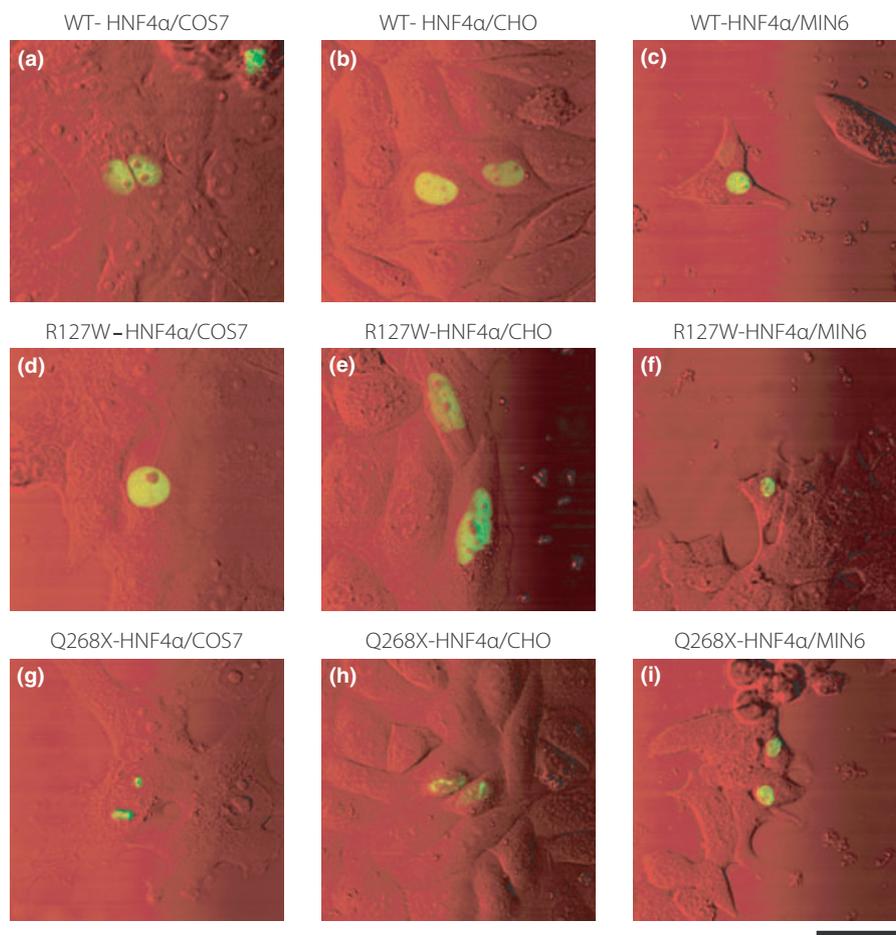
Figure 1 | (a–i) Subcellular localization of wild-type hepatocyte nuclear factor-4α (WT-HNF4α), R127W-HNF4α and Q268X-HNF4α tagged with enhanced yellow fluorescent protein, 48 h after transfection in monkey kidney 7 (COS7), Chinese hamster ovary (CHO) and mouse insulinoma (MIN6) cells.

Cell Culture and Transfection

COS7, CHO, MIN6 and INS-1 cells were cultured in glass-bottomed culture dishes with 2 mL Dulbecco's modified Eagle's medium, containing 10% or 15% fetal bovine serum without or with 72 µmol/L 2-mercaptoethanol, respectively[19,20]. Plasmid DNA was transfected by using FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Fluorescence Microscopic Analysis and Fluorescence Resonance Energy Transfer

For analysis of fluorescence images, cells were observed under a microscope equipped with a silicon intensifier target camera. Using band-pass filters, excitation wavelength was set at 480 ± 10 nm for EYFP and 420 ± 15 nm for ECFP, and emission wavelength was detected at 525 ± 13 and 480 ± 15 nm, respectively. To examine FRET, 420 and 480 nm excitation wavelengths were separated by a 455-nm dichroic mirror. The fluorescence area of an image was calculated from quantitative pixel data and average values were obtained from seven to 10

experiments. The cellular localization of the protein was observed with a confocal laser scanning microscope using a 488-nm argon laser.

Immunohistochemical Analysis

FLAG-tagged proteins were detected immunohistochemically using an anti-FLAG monoclonal antibody (Sigma Aldrich, St Louis, MO, USA) and Cy3-labeled secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in cells permeabilized by 0.1% Triton X-100, and then examined with a confocal microscope.

Statistics

Data are expressed as mean ± SD. One-way analysis of variance was used. When the ANOVA value was significant, comparison between two groups in each experiment was carried out using ANOVA followed by Dunnett's t-test to determine significance. $P < 0.05$ was considered significant.

RESULTS

Subcellular Localization of WT-HNF4α, and R127W and Q268X Mutants of HNF4α (R127W-HNF4 and Q268X-HNF4α) in various cell types

We first examined the cellular distribution of WT-HNF4α and its various mutants. WT-HNF4α tagged with YFP (WT-HNF4α/YFP) was predominantly localized in the nucleoplasm.
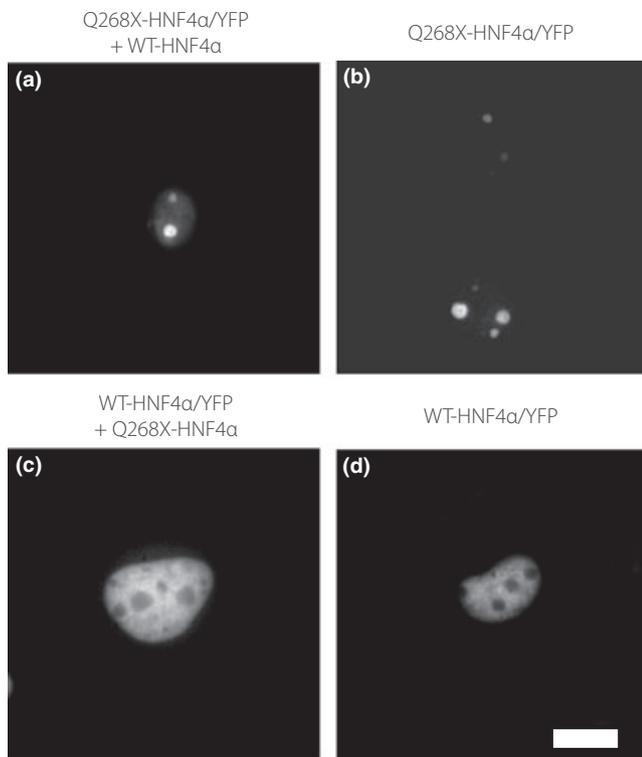
Figure 2 | (a) Nuclear distribution of wild-type hepatocyte nuclear factor-4α (WT-HNF4α)/yellow fluorescent protein (YFP) in monkey kidney 7 (COS7) cells in the presence of enhanced yellow fluorescent protein (EYFP)-free Q268X-HNF4α. (b) Nuclear distribution of WT-HNF4α/YFP in COS7 cells in the absence of Q268X-HNF4α. (c) Distribution of Q268X-HNF4α/YFP in the presence of EYFP-free WT-HNF4α. (d) Distribution of Q268X-HNF4α/YFP in the absence of WT-HNF4α. The deoxyribonucleic acid ratio used for cotransfection of WT-HNF4α:Q268X-HNF4α was 1:2.
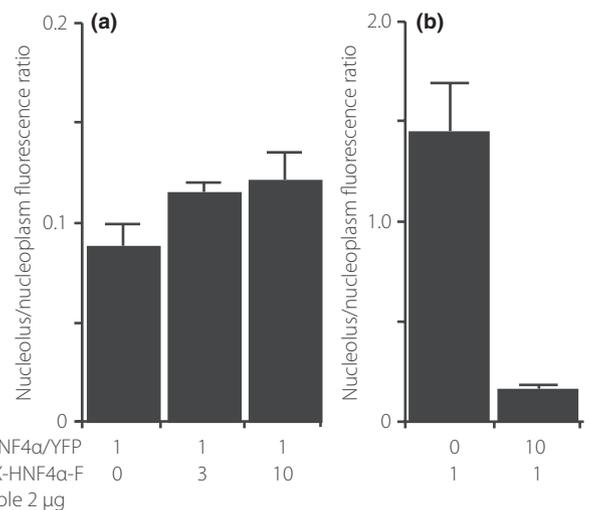
Figure 3 | (a) Fluorescence intensity ratio (F_R) of wild-type hepatocyte nuclear factor-4α (WT-HNF4α)/yellow fluorescent protein (YFP) coexpressed with enhanced yellow fluorescent protein (EYFP)-free Q268X-HNF4α in monkey kidney 7 (COS7) cells transfected with deoxyribonucleic acid (DNA) ratios of 1:0, 1:3 and 1:10. The total amount of DNA was kept at 2 mg. (b) F_R of Q268X-HNF4α/YFP coexpressed with EYFP-free WT-HNF4α after transfection with DNA ratios of 1:0 and 1:10. Fluorescence intensity was obtained in an optical section under confocal microscopy. F_R is presented as the mean ± SD from seven to 10 experiments.

A trace of signals was also observed in the nucleoli of COS7, CHO and MIN6 cells (Figure 1a–c, respectively). Similar distribution was observed with R127W-HNF4α mutant protein tagged with YFP (W127W-HNF4α/YFP (Figure 1d–f). In contrast, truncated Q268X-HNF4α mutant tagged with YFP (Q268X-HNF4α/YFP) was predominantly localized in the nucleoli of COS7 and CHO cells (Figure 1g,h). However, in MIN6 cells, Q268X-HNF4α/YFP was located in the nucleoplasm as well as in nucleoli (Figure 1i). The observation was confirmed by immunohistochemical analysis using the same constructs in which YFP was replaced with FLAG at the Nterminus (data not shown), suggesting that the cellular distribution was not affected by the addition of YFP at the Cterminus.

We then examined how the interaction between WT-HNF4α and Q268X-HNF4α mutant proteins would affect nuclear localization. Q268X-HNF4α, which was distributed in both the nucleoplasm and nucleoli in MIN6 cells (Figure 1i) in contrast to COS7 and CHO cells. Because HNF4α is endogenously expressed in MIN6 cells and also expressed in other insulinoma-derived β-cells[1,20], and is absent in COS7 and CHO cells[21], we hypothesized that the interaction between WT-HNF4α and Q268X-HNF4α would lead to the redistribution of these proteins in the nucleus. To address this issue, WT-HNF4α and Q268X-HNF4α (DNA ratio, 1:2) were cotransfected in COS7 cells. WT-HNF4α was exclusively localized in the nucleoplasm (Figure 2d). However, in the presence of EYFP-free Q268X-HNF4α, WT-HNF4α/YFP was markedly accumulated in nucleoli (Figure 2c) compared with cells with no expression of Q268X-HNF4α (Figure 2d). The effect of Q268X-HNF4α on the nuclear redistribution of WT-HNF4α was further quantified by measuring the fluorescence intensity (F) of YFP within nucleoli and nucleoplasm using a confocal microscope. The fluorescence ratio F_R was 0.042 ± 0.003 for WT-HNF4α/YFP alone (Figure 2), then it increased with cotransfection of YFP-free Q268X-HNF4α dose-dependently with DNA ratios of 1:3 (0.083 ± 0.003) and 1:10 (0.109 ± 0.007; Figure 3). Conversely, at a DNA ratio of

WT-HNF4α/CFP + Q268X-HNF4α/YFP

WT-HNF4α/YFP + Q268X-HNF4α/CFP

WT-HNF4α/CFP + Q268X-HNF4α/YFP

WT-HNF4α/YFP + Q268X-HNF4α/CFP

WT-HNF4α/CFP + Q268X-HNF4α/YFP

WT-HNF4α/YFP + Q268X-HNF4α/CFP

excitation   emission
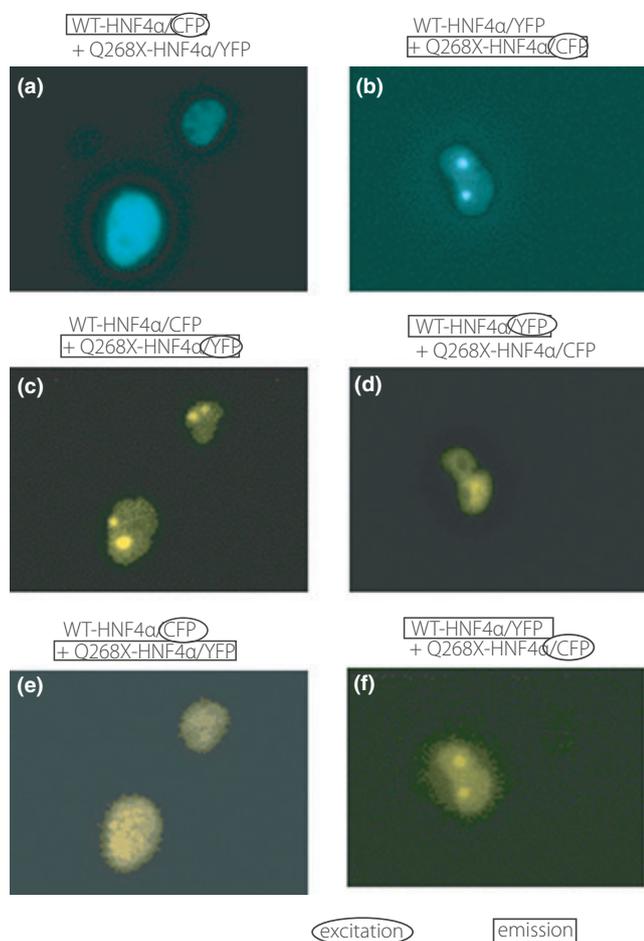
Figure 4 | Direct interaction between Q268X-hepatocyte nuclear factor-4α (HNF4α) and wild-type (WT)-HNF4α in living cells. (a–c) Fluorescence of coexpressed WT-HNF4α/cyan fluorescent protein (CFP) and Q268X-HNF4α (deoxyribonucleic acid ratio 1:2) in monkey kidney 7 (COS7) cells. (d–f) Distribution of coexpressed WT-HNF4α/YFP and Q268X-HNF4α/CFP. (c,f) Fluorescence resonance energy transfer images.
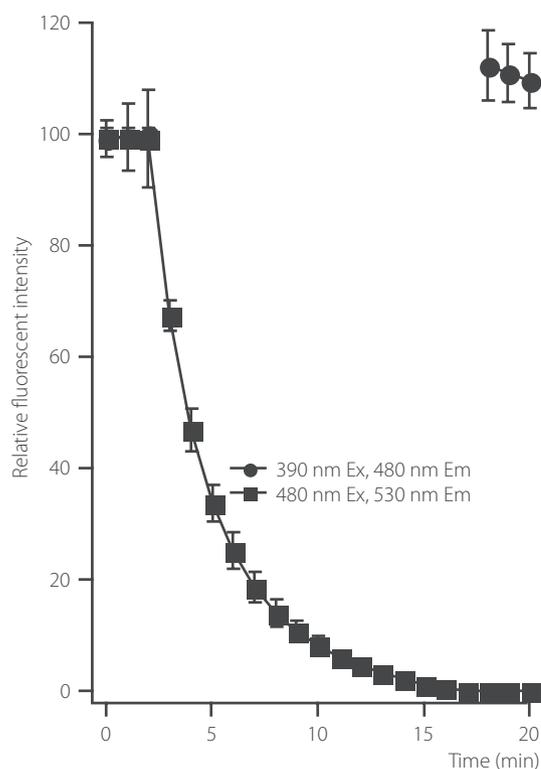
Figure 5 | Evidence for fluorescence resonance energy transfer. Relative fluorescence of the acceptor, Q268X-hepatocyte nuclear factor-4α (HNF4α)/yellow fluorescent protein (YFP; squares), was reduced during repetitive excitation of YFP, whereas that of the donor, wild-type HNF4α/cyan fluorescent protein (CFP; circles), was enhanced after photobleaching of the acceptor.

10:1, the F_R value of 1.58 ± 0.27 with Q268X-HNF4α alone was decreased to 10% (0.152 ± 0.014) when cells were cotransfected with YFP-free WT-HNF4α and Q268X-HNF4α/

YFP DNA (Figure 3). These findings show that the WT-HNF4α and Q268X-HNF4α interaction potentiates the nuclear localization of this protein complex.

Heterodimer Formation of WT-HNF4α and Q268X-HNF4α in Living Cells

The interaction between WT-HNF4α and Q268X-HNF4α in the nuclear redistribution strongly suggests direct binding between these two proteins in cultured cells, as reported previously[12]. To examine this binding, WT-HNF4α/CFP (donor in FRET) and Q268X-HNF4α/YFP (acceptor in FRET) were coexpressed in COS7 cells (DNA ratio, 1:2 Figure 4a,c,e). Figure 4c shows the separated fluorescence of Q268X-HNF4α/YFP in both nucleoli and nucleoplasm on excitation of ECFP, indicating FRET from the WT-HNF4α/CFP to the Q268X-HNF4α/YFP. FRET from ECFP to EYFP was confirmed by bleaching EYFP (Figure 5). FRET was also detected in the reversed condition (Figure 4b,d,f). Mutual FRET indicates that there is a close association between WT-HNF4α and Q268X-HNF4α in forming a heterodimer in the nucleoplasm and nucleoli. FRET from WT-HNF4α/CFP to Q268X-HNF4α/YFP and vice versa showed a similar fluorescent signaling pattern in which the nucleoli were brighter than the nucleoplasm (Figure 4c,f). This is quantified by calculating the average fluorescence in the nucleolus and the nucleoplasm relative to the whole nucleus (Figure 6). Fluorescence through FRET in the nucleolus was prominent compared with that in the nucleoplasm, and the value was nearly 50% between the self-excited

Figure 6 | Averaged fluorescence intensity of wild-type-hepatocyte nuclear factor-4α (WT-HNF4α) and Q268X-HNF4α by fluorescence resonance energy transfer (FRET) in the nucleolus and nucleoplasm relative to fluorescence in the whole nucleus.
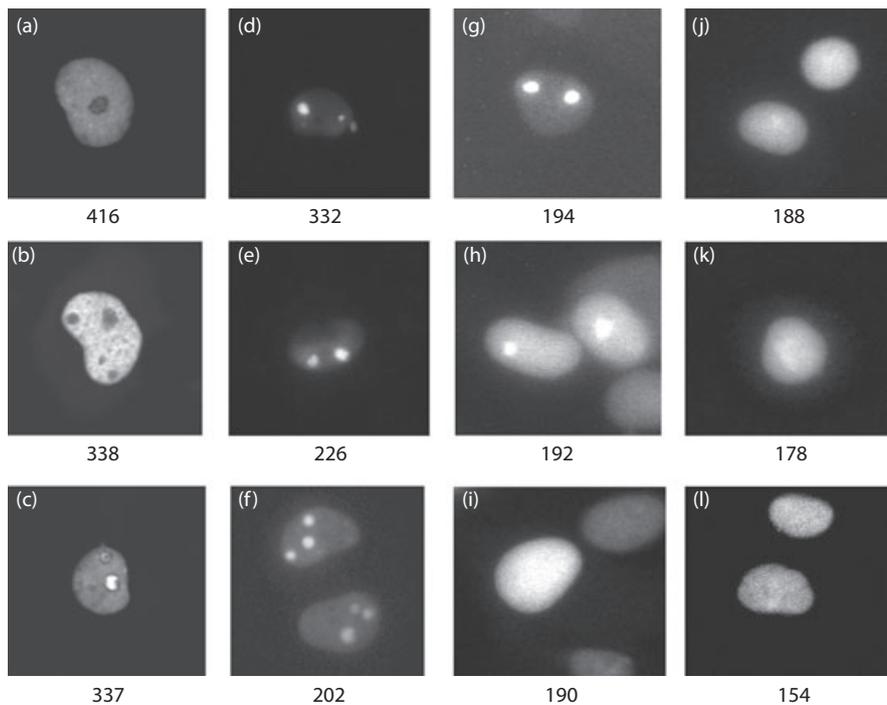
Figure 7 | (a–l) Intranuclear distribution of the indicated deletion mutants tagged with cyan fluorescent protein (CFP) expressed in monkey kidney 7 (COS7) cells. The numbers indicate the extent of amino acid deletions from the C-terminal of HNF4α.

SHP/YFP/COS7

SHP/YFP
+ Q268X-HNF4α-F/COS7

excitation

emission

SHP/YFP
+ Q268X-HNF4α/CFP/COS7

SHP/YFP
+ Q268X-HNF4α/CFP/COS7

SHP/YFP
+ Q268X-HNF4α/CFP/COS7
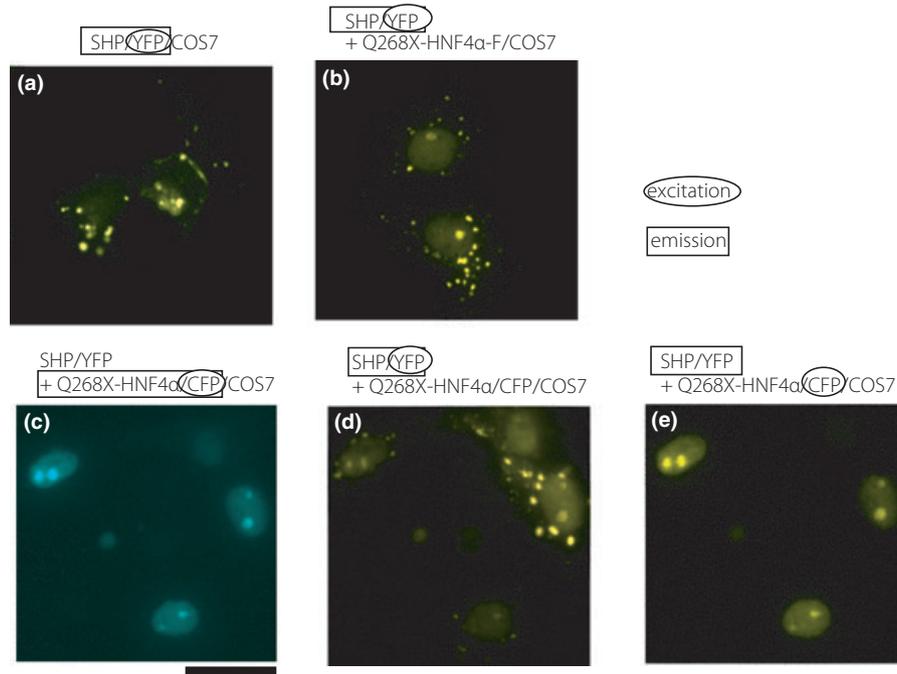
Figure 8 | Direct interaction between Q268X-hepatocyte nuclear factor-4α (HNF4α) and small heterodimer partner (SHP) in monkey kidney 7 (COS7) cells. (a) Expression of SHP/yellow fluorescent protein (YFP) alone. (b) SHP/YFP coexpressed with enhanced YFP-free Q268X-HNF4α (deoxyrobonucleic acid [DNA] ratio 1:1). (c–e) Cotransfection of SHP/YFP and Q268X-HNF4α/CFP (DNA ratio, 1:2).

fluorescence of WT-HNF4α and Q268X-HNF4α in the nucleolus. These findings show that changes in the localization of these molecules might occur after the binding of WT-HNF4α and Q268X-HNF4α to form a heterodimer. Collectively, the predominant localization of HNF4α in the nucleolus might depend on the dimer formation that affects HNF4α function.

Structure Analysis of Truncated HNF4α Mutants

The molecular region that determines the nucleoplasm and nucleolus localization of HNF4α was examined by expressing a series of mutants with deletions in the C-terminus of HNF4α in COS7 cells (Figure 7). There was predominant localization from the nucleoplasm to nucleoli of the I338X and S337X HNF4α mutants. However, any single amino acid replacement with M, A, G or F amino acids in the region from E327 to Q341 in the full-length HNF4α, and deletion of five amino acids from S337 to Q341 did not alter the predominant nucleoplasm localization, as was seen with the WT HNF4α. Furthermore, predominant nucleoli localization of a shorter mutant of HNF4α, A250X, was noted (Figure 7e,l,g). An even shorter HNF4α mutant, R154X, which is a heterozygous mutation found in MODY1[22], showed the nuclear localization ability, but it was distributed in the nucleoplasm as well as the nucleoli (Figure 7l). In contrast, HNF4α mutations with amino acid sequences shorter than W192XHNF4α showed gradual spreading to the nucleoplasm in accordance with the shortness of the amino acid length.

Binding Between SHP and Q268X-HNF4α

Next, we examined the binding of SHP and Q268X-HNF4α, and the effect of this interaction on nuclear localization. SHP-YFP was only present in the cytoplasm of COS7 cells as scattered granular spots (Figure 8a). SHP exists partially in the nucleolus, not only cytoplasm, when cotransfection of Q268X-HNF4α-F occurs (Figure 8b). Cotransfection of twice the amount of Q268X-HNF4α/CFP DNA resulted in the localization of Q268X-HNF4α in the nucleoli and nucleoplasm (Figure 8c), and SHP being in the nucleoli, nucleoplasm and cytoplasm (Figure 8d). FRET from Q268X-HNF4α/CFP to SHP-YFP was observed in the nucleoli and nucleoplasm (Figure 8e). The results show that the binding of SHP-Q268-XHNF4α induces the translocation of this protein complex to the nucleoli.

Structure–Function Analysis of Truncated HNF4α Mutants in Relation to Nuclear Distribution and Dimerization

All results of structure–function analysis of HNF4α are shown in Figure 9, collectively. The binding activity of WT-HNF4α and SHP, detected by FRET, was conserved for deletion mutants, at least up to A250X. In contrast, the R154X mutant lacked binding ability to WT-HNF4α or SHP, as they did not display FRET (data not shown).

DISCUSSION

Although a number of mutations in HNF4α are associated with various diseases, C-terminal truncated mutations in HNF4α are

HNF4α (schematic)	WT-HNF4α	Cellular localization	Binding with SHP	Dimerization with WT-HNF4α
AF-1 DBD Ligand binding/Dimerization NRD	455	Nucleoplasm	Yes	Yes
AF-1 DBD Ligand binding/Dimerization NRD	416	Nucleoplasm	Yes	Yes
AF-1 DBD Ligand binding/Dimerization NRD	390	Nucleoplasm	Yes	Yes
AF-1 DBD Ligand binding/Dimerization	368	Nucleoplasm	Yes	Yes
AF-1 DBD Ligand binding/Dimerization	338	Nucleoplasm	Yes	Yes
AF-1 DBD Ligand binding/Dimerization	337	Nucleolus	Yes	Yes
AF-1 DBD Ligand binding/Dimerization	332	Nucleolus	Yes	Yes
AF-1 DBD Ligand binding	323	Nucleolus	Yes	Yes
AF-1 DBD Ligand binding	268	Nucleolus	Yes	Yes
AF-1 DBD Ligand binding	250	Nucleolus	Yes	Yes
AF-1 DBD — Gradually change of cellular localization according to shorten HNF4α protein mutation	154	Whole nucleus	No	No

Figure 9 | Schematic illustration of full-length wild-type-hepatocyte nuclear factor-4α (WT-HNF4α) and C-terminal deletion mutants, and a summary of experimental results. There was predominant nucleoplasm or nucleolus localization of each mutant tagged with cyan fluorescent protein (CFP) in monkey kidney 7 (COS7) cells. Binding of each mutant with WT-HNF4α or the small heterodimer partner (SHP) was detected by fluorescence resonance energy transfer (FRET). DBD, deoxyribonucleic acid binding site; NRD, negative regulatory domain.

exclusively reported in MODY1 patients. In the present study, we have shown that the Q268X-HNF4α mutant was predominantly localized in the nucleolus when transfected in CHO and COS7 cells in the absence of endogenous HNF4α. The binding of the Q268X-HNF4α mutant to WT-HNF4α was confirmed by FRET, which facilitated the resulting heterodimer to translocate to the nucleolus. A similar pattern of altered subcellular localization to the nucleolus by truncation has been reported that when truncated, murine double minute (MDM) 2 loses the ability to bind its partner p53[23]. HNF4α also binds to p53, suggesting that specific sites might be required for the actions of HNF4α[24,25]. Because endogenous HNF4α can affect the cellular localization of Q268X-HNF4α through heterodimer formation, we used COS7 cells to further determine how the interactions of these proteins contribute to their cellular localization. The use of COS7 cells enabled us to show that the Q268X-HNF4α bound to WT-HNF4α and affected the intracellular localization.

Our systematic analysis by using a series of deletion mutants from the C-terminus of HNF4α showed that a significant change in localization from the nucleoplasm to nucleoli occurred with the I338X and S337X mutants. This result has demonstrated that serine-337 and isoleucine-338 of HNF4α are key amino acids that control the nuclear and subnuclear localization of HNF4α. Interestingly, serine-337 of HNF4α is one of four key amino acids in HNF4α that contributes to protein folding of HNF4α[26]. We also found that any single amino acid substitution at the E327 to Q341 regions in full length HNF4α, as well as the deletion of five amino acids from S337 to Q341, did not alter the predominant nucleoplasm localization, which is evident with WT-HNF4α. Taken together, serine-337 of HNF4α and a region near the C-terminus are required for HNF4α nucleolus localization. HNF4α mutations with amino acid sequences shorter than the W192X-HNF4α mutant showed gradual spreading to the nucleoplasm in accordance with the shortness of the protein length, suggesting an important role of this region. The A250X-HNF4α mutant was also capable of binding and accumulating WT-HNF4α and SHP in the nucleoli, but the R154X-HNF4α mutant did not have binding ability to WT-HNF4α nor to SHP, and mostly resided within the nucleus. Elucidation of the sites that contribute to HNF4α nuclear localization should be important to better understand the pathogenesis of the disease, as changes in HNF4α localization might indicate the early stage of apoptosis.

REFERENCES

1. Yamagata K, Furuta H, Oda N, *et al*. Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature* 1996; 384: 458–460.

2. Furuta H, Iwasaki N, Oda N, *et al*. Organization and partial sequence of the hepatocyte nuclear factor-4 alpha/MODY1 gene and identification of a missense mutation, R127W, in a Japanese family with MODY. *Diabetes* 1997; 46: 1652–1657.

3. Fajans SS. Maturity-onset diabetes of the young (MODY). *Diabetes Metab Rev* 1989; 5: 579–606.

4. Gupta R, Vatamaniuk M, Lee C, *et al*. The MODY1 gene HNF-4alpha regulates selected genes involved in insulin secretion. *J Clin Invest* 2005; 115: 1006–1015.

5. Crossley M, Ludwig M, Stowell KM, *et al*. Recovery from hemophilia B Leyden: an androgen-responsive element in the factor IX promoter. *Science* 1992; 257: 377–379.

6. Sugai M, Umezu H, Yamamoto T, *et al*. Expression of hepatocyte nuclear factor 4 alpha in primary ovarian mucinous tumors. *Pathol Int* 2008; 58: 681–686.

7. Sel S, Ebert T, Ryffel GU, *et al*. Human renal cell carcinogenesis is accompanied by a coordinate loss of the tissue specific transcription factors HNF4 alpha and HNF1 alpha. *Cancer Lett* 1996; 101: 205–210.

8. Tanaka T, Jiang S, Hotta H, *et al*. Dysregulated expression of P1 and P2 promoter-driven hepatocyte nuclear factor-4alpha in the pathogenesis of human cancer. *J Pathol* 2006; 208: 662–672.

9. Ryffel GU. Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *J Mol Endocrinol* 2001; 27: 11–29.

10. Ellard S, Colclough K. Mutations in the genes encoding the transcription factors hepatocyte nuclear factor 1 alpha (HNF1A) and 4 alpha (HNF4A) in maturity-onset diabetes of the young. *Hum Mutat* 2006; 27: 854–869.

11. Jiang G, Nepomuceno L, Hopkins K, *et al*. Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol Cell Biol* 1995; 15: 5131–5143.

12. Bogan AA, Dallas-Yang Q, Ruse MD Jr, *et al*. Analysis of protein dimerization and ligand binding of orphan receptor HNF4alpha. *J Mol Biol* 2000; 302: 831–851.

13. Laine B, Eeckhoute J, Suaud L, *et al*. Functional properties of the R154X HNF-4alpha protein generated by a mutation associated with maturity-onset diabetes of the young, type 1. *FEBS Lett* 2000; 479: 41–45.

14. Sladek FM, Dallas-Yang Q, Nepomuceno L. MODY1 mutation Q268X in hepatocyte nuclear factor 4alpha allows for dimerization in solution but causes abnormal subcellular localization. *Diabetes* 1998; 47: 985–990.

15. Miura A, Yamagata K, Kakei M, *et al*. Hepatocyte nuclear factor-4alpha is essential for glucose-stimulated insulin secretion by pancreatic beta-cells. *J Biol Chem* 2006; 281: 5246–5257.

16. Nishigori H, Tomura H, Tonooka N, *et al*. Mutations in the small heterodimer partner gene are associated with mild obesity in Japanese subjects. *Proc Natl Acad Sci USA* 2001; 98: 575–580.

17. Zhang Y, Soto J, Park K, *et al*. Nuclear receptor SHP, a death receptor that targets mitochondria, induces apoptosis and inhibits tumor growth. *Mol Cell Biol* 2010; 30: 1341–1356.

18. Ogata M, Awaji T, Iwasaki N, *et al*. Nuclear translocation of SHP and visualization of interaction with HNF-4alpha in living cells. *Biochem Biophys Res Commun* 2002; 292: 8–12.

19. Miyazaki J, Araki K, Yamato E, *et al*. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 1990; 127: 126–132.

20. Asfari M, Janjic D, Meda P, *et al*. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 1992; 130: 167–178.

21. Stoffers DA, Ferrer J, Clarke WL, *et al*. Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. *Nat Genet* 1997; 17: 138–139.

22. Lindner T, Gragnoli C, Furuta H, *et al*. Hepatic function in a family with a nonsense mutation (R154X) in the hepatocyte nuclear factor-4alpha/MODY1 gene. *J Clin Invest* 1997; 100: 1400–1405.

23. Lohrum MAE, Ashcroft M, Kubbutat MHG, *et al*. Identification of a cryptic nucleolar-localization signal in MDM2. *Nat Cell Biol* 2000; 2: 179–181.

24. Maeda Y, Hwang-Verslues WW, Wei G, *et al*. Tumour suppressor p53 down-regulates the expression of the human hepatocyte nuclear factor 4a (HNF4a) gene. *Biochem J* 2006; 400: 303–313.

25. Lee YK, Dell H, Dowhan DH, *et al*. The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: two mechanisms for repression. *Mol Cell Biol* 2000; 20: 187–195.

26. Iordanidou P, Aggelidou E, Demetriades C, *et al*. Distinct amino acid residues may be involved in coactivator and ligand interactions in hepatocyte nuclear factor-4alpha. *J Biol Chem* 2005; 280: 21810–21819.