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Evaluation of the effect of the NRF2 and HNF family genes mutations found in human liver cancer on transcriptional activity using mammalian cells

Ocena wpływu mutacji genów z rodziny NRF2 i HNF występujących w ludzkim raku wątroby na aktywność transkrypcyjną w komórkach ssaków

Doctoral thesis

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1. List of publications constituting doctoral dissertation

1. Haque, E.; Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines. Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296
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2. Haque, E.; Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks. Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413
   Impact Factor= 4.096
   Points of Ministry of Science and Higher Education= 70

3. Haque, E.; Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis. Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378
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2. Abbreviations

Human apolipoprotein B	APOB
AT-Rich Interaction Domain 1A	ARID1A
AT-rich interactive domain 2	ARID2
Axin-1 gene	*AXIN1*
Antioxidant response element	ARE
A cellular homolog of the retroviral v-Myc oncogene	MYC
One-way analysis of variance	ANOVA
Basic-region leucine zipper	Bzip
Brahma-related gene 1	BRG1
Catenin Beta 1 gene	*CTNNB1*
CCAAT enhancer-binding protein alpha	CEBPA
Choline- devoid methionine-deficient	CMD
c-Jun N-terminal kinases	JNKs
Cullin 3-RING E3	CUL3 E3
Cluster of differentiation	Cd36
[CREB(cAMP-response-element-binding protein)-binding protein]	CBP
Chemiluminescent EMSA detection	CSPD
4′,6-diamidino-2-phenylindole	DAPI
Diethylnitrosamine	DEN
DNA Binding Domain	DBD
Differentially expressed genes	DEGs
Discs-large domain	DLG
Dulbecco's modified Eagle's medium	DMEM
Electrophoretic Mobility-Shift Assay	EMSA
Esophageal carcinoma	ESCA
Extracellular signal-regulated kinases	ERK
Fetal bovine serum	FBS
Fatty acid-binding protein 4	Fabp4
Gene ontology	GO
Glypican-3	Gpc3
Hepa1-6 mouse hepatoma cells	Hepa1-6 cells
Hepatitis B virus	HBV
Hepatocellular adenomas	HCAs
Hepatitis C virus	HCV
Hepatocellular carcinoma	HCC
Hepatocyte nuclear factor 1A	HNF1A
Hepatocyte nuclear factor 4A	HNF4A
Hexokinase 2	HK2
Hepatic stellate cells	HSCs
Human embryonic kidney cells 293	HEK293
Human hepatoma cell line	HepG2
Human hepatoma cells-7	Huh-7
Immunofluorescence	IFC
International Cancer Genome Consortium	ICGC
Kelch-like ECH-associated protein 1	KEAP1
Kirsten rat sarcoma viral oncogene homolog	K-Ras
Knockdown	KD
Knockout	KO
Kyoto Encyclopedia of Genes and Genomes	KEGG
Lung squamous cell carcinoma	LUSC
liver fatty acid-binding protein	LFABP
Lipoprotein lipase	Lpl
Mammalian target of rapamycin	mTOR

Mammalian target of rapamycin complex 1	mTORC1
Methylenetetrahydrofolate dehydrogenase 1–like	MTHFD1L
microRNA	miR
MAPK or ERK kinases	MEK
Matrix metalloproteinase 9	MMP-9
Maturity-onset diabetes of the young type 3	MODY3
Mitogen-activated protein kinase	MAPK
Microsomal triglyceride transfer protein	MTP
Molecular dynamics	MD
Mouse hepatoma cell	Hepa 1-6
M2 pyruvate kinase	PKM2
Next-generation sequencing	NGS
NRF2-ECH homologies domains	Neh
Nuclear factor erythroid 2–related factor 2	Nrf2
NF-E2-related factor 2	NRF2
Non-alcoholic fatty liver disease	NAFLD
Non-alcoholic steatohepatitis	NASH
Non-immunogenic mouse hepatoma cells	Hepa1-6
Non-small cell lung cancer	NSCLC
Nuclear factor kappa-B	NF-κB
Phosphatidylinositol-3-kinase	PI3K/Akt
Phosphoinositide 3-kinase	P13K
Polymerase chain reaction	PCR
Pit-Oct-Unc (POU) homeodomain	POUh
POU specific	POUs
Putative transforming gene of avian sarcoma virus 17	JUN
Peroxisome proliferator-activated receptor gamma	PPARG
Root mean square fluctuation	RMSF
Reactive oxygen species	ROS
Root-mean-square deviation	RMSD
Standard errors of the means	SEMs
Small interfering RNA	siRNA
Small musculoaponeurotic fibrosarcoma	sMaf
Telomerase reverse transcriptase	TERT
The Cancer Genome Atlas	TCGA
The Library of Integrated Network-Based Cellular Signatures	LINCS
Uterine corpus endometrial carcinoma	UCEC
Visual Molecular Dynamics	VMD
V-raf murine sarcoma viral oncogene homolog B1	BRAF
Western blot	WB
Wingless-related integration site	Wnt
World Health Organization	WHO
Wilms' tumor suppressor gene	WT

3. Abstract

Liver cancer is one of the most troublesome human malignancies. Worldwide, East Asia had the highest liver cancer burden in 2017. The development of liver cancer is influenced by several factors, including chronic hepatitis B (HBV) or C (HCV) virus infection, alcohol intake, diabetes, fatty liver disease, and chronic liver injury. These factors speed up permanent hepatocellular damage, hepatocyte regeneration, inflammation, and genetic alteration. Recently, research focus primarily on investigating the liver cancer genome has identified several transcription factors responsible for somatic mutations in the liver cancer genome. This thesis focuses on the validation of the effect of certain transcription factor mutations from the International Cancer Genome Consortium database of the liver cancer genome, which are mostly related to oxidative stress in the liver and regulating liver homeostasis and organogenesis. Transcription factors regulate gene expression by binding with its target DNA, which influences RNA polymerase activity in a gene-specific manner. Transcription factors regulate their target genes by binding to their target gene promoters. The dysregulation of transcription factors is one of the causes of human cancer. In cancer cells, genes encoding transcription factors are often subjected to genetic alteration or mutations that result in either gain or loss-of-function. This thesis, investigates the functional validation of the mutations found in the functional domain of the nuclear factor erythroid2-related factor 2 (NRF2) and hepatocyte nuclear factor 1A (HNF1A) transcription factors responsible for hepatocellular carcinoma (HCC) development.

The stress-related transcription factor NRF2 regulates the expression of a battery of cytoprotective genes containing antioxidant response element (ARE) in their promoter sequences. Strong evidence exists that enhanced NRF2 activity can prevent cancer and many other diseases with great therapeutic potential, in which oxidative stress is crucial for pathogenesis. Conversely, the aberrant activation of NRF2 has been found in different cancers and has been well-studied. Next-generation sequence analyses have shown that mutations in NRF2 are found in several cancers; these mutations cause aberrant NRF2 activity and are associated with cancer progression due to the development of resistance to chemotherapy and poor prognosis. However, the effect of NRF2 mutations in liver cancer development remains unknown. My study focuses on the aberrant transcriptional activity of NRF2 mutations found in Japanese liver cancer patients located at the functional DLG domain of NRF2. I used the liver carcinoma cell lines to perform the reporter assay to test the hypothesis that mutation causes aberrant transcriptional activity. The transcriptional activity of NRF2 mutations is independent of Kelch-like ECH-associated protein 1 (KEAP1), presumably because NRF2 mutations disturb proper NRF2–KEAP1 binding and block the KEAP1-mediated degradation of NRF2. Additionally, mutations upregulate the transcriptional activity of NRF2 target gene MMP9, suggesting that the mutation-derived gain-of-function of NRF2 is important for liver tumour progression. Interestingly, the ectopic overexpression of oncogenic BRAF (V-raf murine sarcoma viral oncogene homolog B1) and its mutation causes the aberrant transcriptional activity of NRF2 and its mutations on both the ARE and MMP9 promoter; this highlights the synergistic effect of both NRF2 and BRAF mutations for aberrant transcriptional activity. As such, the high activity of NRF2 mutations in HCC with BRAF mutations warrants further exploration of the potential diagnostic, prognostic, and therapeutic utility of this pathway in HCC.

Another part of my study focused on the functional analysis of mutations of HNF1A transcription factors, which are crucial in the development of and the maintenance of the normal homeostasis in a variety of tissues, including liver, kidney, and small intestine. Even though the HNF1A is a well-established tumour suppressant, the functional importance of its mutations is yet to be elucidated. In mice, the deletion of HNF1A is known to lead to the development of NAFLD (Non-alcoholic fatty liver disease)-HCC; these mice also developed fatty liver, non-alcoholic steatohepatitis, and liver tumours characterised by collagen deposition and showed an intense expression of glypican-3, a diagnostic marker for HCC. I thus hypothesised that any loss-of-function variation to the gene structure or mutation can cause aberrant gene expression to trigger liver cancer development, along with disrupted transcriptional networks in liver cells. From the International Cancer Genome Consortium database of

cancer genomes, I found that several HNF1A mutations located in the functional POU (Pit-Oct-Unc homeodomain) domain suppressed HNF4A promoter activity. Moreover, I also found the disrupted binding of HNF1A to its target HNF4A promoter, without any effect on nuclear localisation. It has been well documented that HNF1A activates HNF4A via the HNF4A promoter, which then activates HNF1A transcription. My results suggest that the decreased transcriptional activity of HNF1A mutants is due to impaired DNA binding. Through structural simulation analysis, I found that mutation was likely to affect DNA interaction by inducing large conformational changes in the N-terminal region of HNF1A. These results suggest that the POU-domain mutations of HNF1A downregulate HNF4A gene expression. Therefore, to mimic the HNF1A mutation phenotype in transcriptional networks, we performed the siRNA (small interfering RNA)-mediated knockdown of HNF4A. Through RNA-Seq data analysis for the HNF4A knockdown, I found that downregulated genes were related to lipid and cholesterol metabolism pathways, which are implicated in HCC development.

In summary, my thesis has shown the aberrant or disrupted transcriptional activity of NRF2 and HNF1A mutations, respectively, which could be responsible for HCC progression. Structural analysis of the mutation revealed that mutation causes structural hindrance and decreases binding affinity. Furthermore, the functional analysis of NRF2 and HNF1A mutations revealed that gain- and loss-of-function can trigger HCC development through dysregulated transcriptional networks.

4. Streszczenie

Rak wątroby jest jednym z najbardziej agresywnych nowotworów u ludzi. Geograficzne największa liczba odnotowanych przypadków raka wątroby w 2017 roku dotyczyła Wschodniej Azji. Na rozwój raka wątroby wpływa wiele czynników, w tym m.in.: przewlekłe zakażenie wirusem zapalenia wątroby typu B (HBV) lub C (HCV), nadużywanie alkoholu, cukrzyca, choroby stłuszczeniowe wątroby oraz rózne przewlekłe choroby wątroby. Czynniki te wzmagają trwałe uszkodzenie komórek wątrobowych, zaburzając regenerację hepatocytów, indukując stany zapalne oraz mutacje genetyczne. Ostatnio wiele badań koncentruje się na badaniu genomu raka wątroby i identyfikowaniu specyficznych somatycznych mutacji czynników transkrypcyjnych w genomie raka wątroby. Prezentowana praca skupia się na walidacji wpływu niektórych mutacji czynników transkrypcyjnych z bazy danych ICGC genomu raka wątroby, głównie związane ze stresem oksydacyjnym w wątrobie jak i te które regulują homeostazę wątroby i organogenezę. Czynniki transkrypcyjne regulują ekspresję genów poprzez wiązanie się z docelową sekwencją DNA, wpływając na aktywność polimerazy RNA w sposób specyficzny dla danego genu. Czynniki transkrypcyjne regulują ekspresję genów docelowych, w szczególności też wiążąc się z promotorami genów docelowych. Zaburzenia prawidłowego działania czynników transkrypcyjnych są jedną z przyczyn rozwoju nowotworów u ludzi. W komórkach nowotworowych geny kodujące czynniki transkrypcyjne często podlegają zmianom genetycznym, mutacjom, które prowadzą do nabywania lub utraty specyficznych funkcji dotyczących regulacji ekspresji genów. Niniejsza praca ma na celu charakterystyką i walidację funkcjonalną wybranych mutacji znajdujących się w funkcjonalnej domenie czynników transkrypcyjnych NRF2 i HNF1A potencjalnie odpowiedzialnych za rozwój raka wątrobowokomórkowego (HCC).

Czynnik transkrypcyjny związany ze stresem „nuclear factor erythroid 2-related factor 2" (NRF2) reguluje ekspresję zestawu genów cytoprotekcyjnych zawierających element odpowiedzi antyoksydacyjnej (ARE) w swoich sekwencjach promotorowych. Istnieją dowody na to, o istotnym potencjale terapeutycznym zwiększonej aktywność NRF2 , która to może zapobiegać nowotworom oraz wielu innym chorobom, w których stres oksydacyjny ma kluczowe znaczenie dla ich patogenezy. Natomiast, nieprawidłowa aktywacja NRF2 została zidentyfikowana i dobrze zbadana w przebiegu różnych nowotworów . Ponadto, analizy oparte na sekwencjonowaniu nowej generacji wykazały, że mutacje w genie NRF2 są identyfikowane w kilku rożnych nowotworach. Mutacje te są przyczyną nieprawidłowej aktywność NRF2 i są też związane z progresją nowotworu w tym poprzez rozwój oporności na chemioterapię w konsekwencji zwieszając złe rokowania dotyczące efektywności podjętego leczenia. Jednak wpływ mutacji NRF2 na rozwój nowotworu wątroby pozostaje niewyjaśniony. Niniejsze badania koncentrują się na charakterystyce zaburzeń aktywności transkrypcyjnej spowodowanej pojawieniem się mutacji w genie NRF2 identyfikowanych w raku wątroby u japońskich pacjentów, a zlokalizowanych w funkcjonalnej domenie DLG NRF2. Linie komórkowe nowotworu wątroby wykorzystano do wykonania tzw. testu reporterowego w celu przetestowania hipotezy zakładającej, że specyficzna mutacja powoduje nieprawidłową aktywność transkrypcyjną. Aktywność transkrypcyjna spowodowana mutacją NRF2 jest niezależna od KEAP1, prawdopodobnie dlatego, że mutacje w NRF2 zaburzają prawidłowe wiązanie NRF2-KEAP1 przez co blokują degradację NRF2, w której pośredniczy KEAP1. Dodatkowo, mutacje zwiększają aktywność transkrypcyjną genu docelowego NRF2 MMP9, co wskazuje, że wzmocnienie funkcji NRF2 spowodowane mutacją może mieć istotny wpływ na progresję nowotworu wątroby. Co ciekawe, ektopowa nadekspresja onkogennego genu BRAF i jego zmutowanego wariantu powoduje nieprawidłową aktywność transkrypcyjną genu NRF2 jak i jego zmutowanej formy zarówno na promotorze ARE, jak i MMP9, podkreślając synergistyczny efekt obu mutacji w genach NRF2 i BRAF na nieprawidłową aktywność transkrypcyjną. W związku z tym wnioskuje się, że wysoka aktywność wariantu zmutowanego NRF2 w HCC wraz z wariantami zmutowanymi w genu BRAF stanowi potencjalne narzędzie o wysokiej użyteczności diagnostycznej, prognostycznej i terapeutycznej tego szlaku w HCC co uzasadnia dalsze badania zmian występujących w tych genach.

Kolejna część prezentowanych przeze mnie badań dotyczyła funkcjonalnej analizy mutacji czynników transkrypcyjnych które odgrywają kluczową rolę w rozwoju i utrzymaniu prawidłowej homeostazy w różnych tkankach/organach, w tym w wątrobie, nerkach i jelicie cienkim, w szczególności roli hepatocytowego jądrowego czynnika 1α (HNF1A). Chociaż HNF1A jest dobrze znanym w terapii czynnikiem hamującym rozwój nowotworów, funkcjonalne znaczenie jego mutacji nie zostało jeszcze w pełni wyjaśnione. Wiadomo, że u myszy, że delecja w genie HNF1A prowadzi do rozwoju NAFLD-HCC; u myszy takich rozwijają się również nowotwory związane ze stłuszczeniem wątroby, NASH, i nowotwory wątroby charakteryzujące się odkładaniem kolagenu, oraz intensywną ekspresją glipikanu-3 (Gpc3), jako markera diagnostycznego dla HCC. W związku z tym założono hipotezę, że jakakolwiek utrata funkcji w strukturze tego genu lub jego mutacja może spowodować nieprawidłową ekspresję genów prze niego kontrolowanych, wywołując rozwój nowotworu wątroby poprzez zakłócanie sieci transkrypcyjnych w komórkach wątroby. Na podstawie weryfikacji genomowej nowotworowej bazy danych, International Cancer Genome Consortium (ICGC) stwierdzono, że kilka mutacji w genie HNF1A zlokalizowanych w funkcjonalnej domenie POU posiada właściwości hamujące aktywność promotora HNF4A. Ponadto stwierdzono, że zaburzenia wiązania HNF1A z jego docelowym promotorem HNF4A nie ma żadnego związku z jego lokalizację jądrową. Jest też dobrze udokumentowane zjawisko, że czynnik HNF1A aktywuje HNF4A poprzez promotor HNF4A, który następnie aktywuje transkrypcję czynnika HNF1A. Uzyskane w toku niniejszych badań wyniki sugerują, że zmniejszona aktywność transkrypcyjna zmutowanej formy genu HNF1A wynika z upośledzenia wiązania do DNA. Poprzez analizę symulacji strukturalnej odkryto, że mutacja ta prawdopodobnie oddziałuje na interakcję z DNA poprzez wywołanie dużych zmian konformacyjnych w regionie N-końcowym genu HNF1A. Wyniki przeprowadzonych wskazują też, że mutacje HNF1A w domenie POU obniżają ekspresję genu HNF4A. Dlatego, aby celem weryfikacji naśladującej wpływ mutacji HNF1A na sieci transkrypcyjne, przeprowadzono knockdown (KD) HNF4A za pośrednictwem siRNA. Dzięki analizie transkryptomicznej opartej na RNA-Seq dla wariantów HNF4A KD odkryto, że geny o obniżonej ekspresji są powiązane ze szlakami metabolizmu lipidów i cholesterolu, i są zaangażowane w rozwój nowotworu wątrobowokomórkowego (HCC).

Podsumowując, przedstawione przeze mnie badania w pracy doktorskiej wykazały nieprawidłową lub zaburzoną aktywność transkrypcyjną specyficzną dla mutacji w genach NRF2 i HNF1A, które mogą też być odpowiedzialne za progresję HCC. Analiza strukturalna wykazała, że mutacja powodowała zmiany strukturalne stanowiące przeszkodę do wiązania przez co zmniejszało się powinowactwo czynników do miejsca docelowego. Ponadto, analiza funkcjonalna mutacji NRF2 i HNF1A wykazała, że wzmocnienie jak i utrata funkcji tych czynników poprzez rozregulowane sieci transkrypcyjne może przyczynić się do rozwój HCC.

5. Introduction

5.1 Liver cancer epidemiology

Cancer ranks as a leading cause of death overall and is the second leading cause of death before the age of 70 years in 112 countries according to the World Health Organization (Sung et al., 2021). Amongst cancers, liver cancer is one of the most troublesome human malignancies, with an annual incidence of around 600,000 worldwide (https://doi.org/10.3322/canjclin.55.2.74). Amongst different types of liver cancer, hepatocellular carcinoma (HCC) is the sixth most common malignancy of the liver (http://gco.iarc.fr/) and the eighth leading cause of cancer-related deaths in Europe (http://gco.iarc.fr/today). The highest incidence of HCC is in Asia and Africa; this difference occurs primarily due to hepatitis B (HBV) viral infections, which are less predominant in developed nations (Di Bisceglie, 2009). Nevertheless, increasing numbers of liver cancer patients have been observed in Europe and the USA (Bosch et al., 2004), (McGlynn et al., 2015).

5.2. HCC etiology

HCC generally progresses in the background of chronic liver inflammation and cirrhosis (El-Serag, 2011). This chronic inflammation contributes to hepatocarcinogenesis. Common risk factors for liver inflammation and subsequent cirrhosis include chronic HBV and hepatitis C (HCV) viral infections and excessive alcohol consumption. Obesity, metabolic diseases, and smoking are also major risk factors for liver inflammation which can lead to cell transformation. HCV can induce HCC progression through several mechanisms. The HBV genome contains circular DNA that is transformed into covalently closed circular DNA; this can result in transcriptional activation and translation of viral proteins, of which the HBx viral protein is associated with the development of liver cancer (Ivanov et al., 2017). HBx can initiate signalling cascades through the activation of kinases such as MAPKs (Mitogen-activated protein kinase) and JNKs (c-Jun N-terminal kinases) (Bouchard & Schneider, 2004). HCV and HBV- driven liver carcinogenesis depends on several factors, but a key factor underlying the oncogenic transformation of single viral proteins is their ability to induce oxidative stress (Bartosch et al., 2009), (Ivanov et al., 2013). Additionally, the oxidative and inflammatory microenvironment is known to be one of the driving forces of developing fibrosis. In regions where viral exposure is not common, a more frequent contributor to HCC is alcohol consumption. Alcohol consumption greater than 80g/day for more than 10 years can augment the risk of HCC development (Morgan et al., 2004). Excessive alcohol leads to hepatic steatosis and subsequently to HCC (Bellentani et al., 1997). Alcohol consumption during chronic HCV infection doubles the risk for HCC, as compared with the risk in HCV alone. Moreover, a synergistic interaction occurs between alcohol and HCV in the development of HCC (Morgan et al., 2004). Diabetes and metabolic syndrome such as obesity, diabetes, anf fatty liver disease, are also HCC risk factors more common in developed nations. Fatty liver disease presents the highest risk (Davila et al., 2005), (Lai et al., 2006)). Moreover, iron deposition in the liver is a major contributors to non-alcoholic steatohepatitis (NASH) and the progression to HCC (Starley et al., 2010). Although etiological factors are responsible for most cases of HCC, the molecular pathogenesis for developing HCC is not completely understood.

5.2.1 Molecular mechanism of HCC progression

The molecular mechanism of hepatocarcinogenesis consists of a multifactorial process in which several pathways can cooperate (Llovet et al., 2021). Among them, the regulators of cell proliferation and survival, dysregulation in tumour suppressor genes, lipid metabolism, autophagic disruption stand out (Inami et al., 2011), (Tward et al., 2007), (Beloribi-Djefaflia et al., 2016), (Alves et al., 2011). As such, different causes underlying hepatocarcinogenesis may induce various oncogenic mechanisms in the liver (Ho et al., 2016). Several studies have revealed that the PI3K/AKT/mTOR (phosphatidylinositol-3-kinase/mammalian target of rapamycin complex 1) and WNT/β (wingless-related integration site)-catenin pathways are critical in the development of HCC (Semela et al., 2007), (Diniz et al., 2020), (Tao et al., 2014)). Mouse models of DEN/CCl4-induced hepatocarcinogenesis are often accompanied with the significant upregulation of liver-specific nuclear factor erythroid 2-related factor 2(NRF2) , NF-κB,

TGF-β1/Smad3 signalling (Mahmoud et al., 2017), (Ngo et al., 2017)) and the reactivation of foetal liver genes, glypican-2, Afp, Slpi, Spink3, and Abcd2 (Ngo et al., 2017), (Chen et al., 2015), (Z. N. Lu et al., 2020). Moreover, NFR enhances the expression of certain metabolic enzymes (G6pd, Pgd, Taldo) required for cell proliferation (Ngo et al., 2017), and DEN-induced HCC rats have shown the downregulation of the tumour suppressor gene HNF4A with the reduction of E-cadherin. However, the expression of vimentin was notably increased (Ning et al., 2010). The somatic mutations in the tumour suppressor gene hepatocyte nuclear factor 1A (HNF1A) in HCC weaken the tumour suppressor function of HNF1A and may play a role in HCC development through a distinct pathway independent of HCC with β-catenin mutations (Tward et al., 2007). The intriguing feature of cancer cells is their metabolic reprogramming, characterised by high glycolysis and lipogenesis (Hanahan & Weinberg, 2011). Multiple lines of evidence suggest that the disruption of the lipid metabolic pathways reduces tumour progression and can offer new avenues for cancer treatment (Beloribi-Djefaflia et al., 2016)). The peroxisome proliferator-activated receptor gamma is a master regulator of lipid uptake, is significantly increased in HCC, and affects metabolic rearrangements and liver tumorigenesis via the transcriptional regulation of hexokinase 2 and M2 pyruvate kinase (Patitucci et al., 2017), (Panasyuk et al., 2012). Moreover, the hydrodynamic gene delivery system in mice revealed novel crosstalk between aberrant lipogenesis and cholesterol biosynthesis pathways in the progression of HCC (Che et al., 2020). Furthermore, the highly activated hepatic stellate cells are known to induce hepatic fibrosis through the IL-6 and TNFA-induced expression of miR-21 and miR-146a in the hepatocytes and thus promote tumour development (Qian et al., 2015). Increasing numbers of studies have shown that autophagy greatly affects HCC (S. Yang et al., 2019). Interestingly, it has been shown that high p62, an autophagy receptor and signalling protein, induces HCC pathogenesis by accelerating the survival of HCC-initiating cells (Umemura et al., 2016). It has been reported that the loss of Atg7 develops hepatocellular adenoma, accompanied by the aberrant accumulation of p62 followed by NRF2 activation (Inami et al., 2011). Furthermore, high levels of p62 expression activate NRF2 and mTORC1 in HCC (Umemura et al., 2016). Despite the frequent activation or disruption of these signalling pathways in HCC, the genetic alterations or mutations found in HCC are also responsible for HCC pathogenesis.

5.2.2 Genetic alteration in hepatocellular carcinoma

Several genetic alterations have been discovered to be associated with HCC progression and have been utilised for prognosis. Approximately 18% of TP53 mutations have been identified in HCC patients (Cleary et al., 2013). p53 mutation has an unfavourable impact on overall survival, and I thus an indicator of poor prognosis for HCC (Zhan et al., 2013). It has been reported that HCV-infected HCCs with mutant TP53 significantly express cell cycle-related genes (CCNG2, BZAP45) and cell proliferation-related genes (SSR1, ANXA2, S100A10, and PTMA). Thus, mutant TP53 tumours develop higher malignant potentials (Hussain et al., 2007). Telomerase reverse transcriptase (TERT) promoter mutation has been identified in 63% of HCC. Two hot spot mutations (124G>A and 146G>A) of TERT promoter were identified in 15 out of 24 HCC cell lines (Nault et al., 2013), upregulating the expression of telomerase (Pezzuto et al., 2017). In normal liver tissue, TERT regulates somatic cells'' lifespan, whereas in HCC, it is associated with uncontrolled cell proliferation (Nault & Zucman-Rossi, 2016). Genetic alterations to the WNT-signalling components *CTNNB1* (catenin Beta 1 gene) and *AXIN1*(*Axin-1 gene*) have also been found in HCC. CTNNB1 mutations are linked with WNT pathway activation and have been associated with large tumours, and the invasion and metastases potential of tumours (Cleary et al., 2013).The identification of loss - of - function mutations in the ARID1A 9 (AT-Rich Interaction Domain 1A) and ARID2 (AT-rich interactive domain 2) genes involved in chromatin organisation and regulation suggests that they have tumour-suppressive roles in HCC. Mutations in these genes might affect the base substitution pattern by changing chromatin structure (Fujimoto, Furuta, et al., 2016). Another known tumour suppressor HNF4A gene mutation was found in the Zn-finger DNA-binding domain, and it has been proposed as a pathogenic mutation. Moreover, low HNF4A expression has been associated with a worse prognosis in liver cancers (Taniguchi et al., 2018). The NF-E2-related factor 2 (NRF2) transcription factor is activated by oxidative stress; recent studies have found that NRF2 is recurrently mutated in HCC (McMahon et al., 2003), (Fujimoto, Furuta, et al., 2016). It has been demonstrated that somatic mutations occur in the

coding region of NRF2 and are associated with poor prognoses (Kerins & Ooi, 2018), (Zhang et al., 2015). Unlike those mutations with relatively low incidences, HNF1A mutations are poorly studied genetic mutations found in HCC (Ding et al., 2018). RNA-Seq data from liver cancer patients has also shown that the expression of HNF4A, HNF1A, and APOB mRNA are significantly correlated (Taniguchi et al., 2018).

5.3 Nuclear factor erythroid 2-related factor 2 (NRF2)

NRF2 is a transcription factor that belongs to the cap "n" collar (CNC) subfamily of basic-region leucine zipper (bZIP) transcription factors (Wang et al., 2013), which regulate the gene expression of antioxidant proteins and detoxification enzymes that protect against oxidative damage (Wang et al., 2013). NRF2 protein possesses seven conserved NRF2-ECH homologies (Neh) domains. The Neh1 domain contains the CNC-bZIP region, via which NRF2 dimerises with its dimerization partners, the small musculoaponeurotic fibrosarcoma (sMaf) proteins (MAFF, MAFG, MAFK) (Wang et al., 2013). The N-terminal region contains the highly conserved Neh2 domain. Which contains two highly conserved amino acid sequences, the DLG and ETGE motifs. Neh2 domain allows NRF2 for binding to its cytosolic repressor Kelch-like ECH-associated protein 1 (KEAP1) through the DLG (discs-large domain) and ETGE motifs, which negatively regulate the transcriptional activity of NRF2. (Wang et al., 2013). The C-terminal Neh3 domain harbours transactivation activity and functions in NRF2 protein stability (Katoh et al., 2001), (Nioi et al., 2005), (Sekine et al., 2016). The N-terminal Neh4 and Neh5 transactivation domains bind to CBP (REB [cAMP-response-element-binding protein]-binding protein) and Brahma-related gene 1 for transcription (Katoh et al., 2001), (Zhang et al., 2006). Neh6 is a serine-rich region target for E3 ubiquitin ligase β-TrCP which leads to the proteasomal degradation of NRF2 (Rada et al., 2011). Alternatively, NRF2 repression is accomplished by interactions of Neh7 with the DNA-binding domain of retinoid X receptor α (Wang et al., 2013). NRF2 functions in a complex regulatory network and performs a pleiotropic role in the regulation of metabolism and immune responses (He et al., 2020).

5.3.1 Oxidative stress and NRF2-KEAP1 signalling

The NRF2-sMaf complex binds to the specific DNA consensus element, known as the antioxidant response element (ARE 5′-TGACXXXGC-3′). The ARE sequence was initially identified as *cis*-regulatory elements for the NQO1 and Gst genes (Friling et al., 1992), (Rushmore et al., 1991), (Telakowski-Hopkins et al., 1988). Following this, the list of proteins that are encoded by the ARE gene array were expanded (Hayes & Dinkova-Kostova, 2014). Under normal conditions, NRF2 is maintained at a very low intracellular concentration through its association with KEAP1 and the Cul3 E3 ligase (Kobayashi et al., 2004). In the presence of oxidative or electrophilic stresses, the KEAP1-mediated proteasomal degradation of NRF2 is hampered and leads to the NRF2-mediated transcription of various genes. The N-terminal region of NRF2 contains the highly conserved Neh2 domain, which negatively regulates the transcriptional activity of NRF2 (Katoh et al., 2005). KEAP1-null animals show postnatal lethality because NRF2 is constitutively accumulated in the nucleus, which results in severe hyperkeratosis in the oesophagus and forestomach (Wakabayashi et al., 2003), and this phenotype is not only reversed by the concomitant disruption of *Nfe2l2* (Wakabayashi et al., 2003). These results suggest that KEAP1 acts upstream of NRF2 in response to oxidative stress. Moreover, many studies have revealed that the loss of NRF2-KEAP1 causes tumour development of multiple cancer types (Shibata et al., 2008) Notably, the deletion of Exon2 in NRF2, which reduces interaction with KEAP1, has been reported to causes tumour development in liver cancer (Goldstein et al., 2016). Taken together, a tightly regulated balance of NRF2 and KEAP1 interaction is essential to protect cells or tissues from oxidative stress, and the failure of this mechanism (e.g., mutations of critical amino acids) triggers cancer development.

5.3.2 Aberrantly activated NRF2 and its target genes and their effect on HCC

The elevated expression of NRF2 target genes has been reported to confer advantages in terms of stress resistance and cell proliferation in both normal and cancer cells (Ohta et al., 2008). Moreover, NRF2 is aberrantly upregulated in HCC and promotes the invasion of HCC through regulating the expression of MMP9 and BCl-xL (Zhang et al., 2015). A strong correlation between NRF2 and the PI3K-Akt signalling pathway has been found to induce metabolic gene expression in the liver, as well as increased hepatocyte proliferation (Mitsuishi et al., 2012). The Cancer Genome Atlas (TCGA) data has shown that Methylenetetrahydrofolate dehydrogenase 1–like (MTHFD1L), to be involved in the folate cycle regulation. When significantly overexpressed in HCC, MTHFD1L provides nutrition to the cells by supplying metabolites for NADPH and DNA synthesis. The *MTHFD1L* promoter has three ARE sequence elements and is transcriptionally controlled by NRF2. The genetic knockdown (KD) of either *NRF2* or *MTHFD1L* can inhibit liver cancer cell proliferation by altering the metabolic program, and sensitising HCC cells to sorafenib treatment (Lee et al., 2017). Furthermore, in KEAP-KD mice, NRF2 is constitutively activated when fed with HFD and has exhibited greater lipogenic gene (Ppar-G, Steroyl CoA desaturase [Scd1], Fatty acid-binding protein 4 [Fabp4], Lipoprotein lipase [Lpl], and Cluster of differentiation [Cd36]) expression, inflammation, and increased hepatic steatosis (More et al., 2013). A recent CRISPR/Cas9 genome-wide screening study demonstrated that KEAP1 deletion causes aberrant NRF2 activity. The induction of NRF2-target gene *NQO1*, *GPX2,* and *TXNRD1* was also observed (Zheng et al., 2019). Interestingly, oncogenic gene mutations such as K-RasG12D and, B-RafV619E also enhanced the transcription of NRF2 with elevated NRF2 target gene expression and lowered intracellular ROS (Reactive Oxygen Species) (DeNicola et al., 2011). As such, it is assumed that aberrant transcriptional activity of NRF2 induced by high expression or mutation of NRF2 may lead to pathogenesis in combination with other factors.

5.3.3 NRF2 mutations in HCC

The International Cancer Genome Consortium (ICGC) database has identified somatic mutations of NRF2 in different cancers (Kerins & Ooi, 2018), (Shibata et al., 2008), (Fujimoto, Furuta, et al., 2016)). Whole-exome sequencing has identified 6.4% of the somatic mutations in *NRF2* in HCC patients (Guichard et al., 2012). Notably, these mutations were mostly located within the DLG and ETGE motifs, which provide NRF2 with gain-of-function activity in various cancer types (Kerins & Ooi, 2018), (Shibata et al., 2008), (Ngo et al., 2017). Interestingly, mutations in *KEAP1* and *NRF2* are mutually limited and rarely occur in the same cancer types ("Comprehensive genomic characterization of squamous cell lung cancers," 2012), and the overlapping *NRF2*/*KEAP1* mutations are associated with a constant NRF2 activation phenotype (Kerins & Ooi, 2018), (Shibata et al., 2008). Moreover, NRF2 gain-of-function mutations are one of the major contributors of HCC (Ngo et al., 2017), (Orrù et al., 2018). In an experimental rat model of hepatocarcinogenesis, it was found that the *NRF2* gene was recurrently mutated or persistently activated during the early stage of the tumorigenic process (Orrù et al., 2018). Thus, NRF2 is critical in the initiation of HCC and is required for the development of preneoplastic lesions. NRF2, DLG, and ETGE mutations lose the interactions with KEAP1, then localised into the nucleus and exert target gene (NQO1, Gclc, and Gsta4) activation (Zavattari et al., 2015). This suggests that *NRF2* mutations can enhance NRF2 transcriptional activity. Mutation in the DLG motif of NRF2 induces pentose phosphate pathway enzyme transcription, which is required for cell growth and proliferation (Ngo et al., 2017). Overall, these findings suggest that both the *NRF2* DLG and ETGE mutations induce aberrant NRF2 activity and may induce HCC through NRF2 ARE pathway activation. As such, many possible pathways appear to trigger liver cancer via aberrant NRF2 transcriptional activity of NRF2 mutations, and further phenotypic validation of the roles of these mutations in liver cancer development merits investigation.

5.4 Hepatocyte nuclear factor 1 alpha (HNF1A)

The HNF1A gene is located on the long (q) arm of chromosome 12 at position 24.31 (Colclough et al., 2013). HNF1A, which is expressed in the liver, small intestine, and kidney (Blumenfeld et al., 1991), is a highly diverged homeoprotein that is critical for the transcription of many hepatocyte-specific genes, including albumin (Blumenfeld et al., 1991). The HNF1A protein is composed of three functional

domains: N-terminal dimerisation domain (amino acids 1-31), a central DNA Binding Domain (DBD, homeodomain amino acids 100 - 279), and a C-terminal transactivation domain (amino acids 280 - 631) (Teeli et al., 2021). The DBD is composed of a POU (Pit1, Oct1, and Unc1)-homeodomain (POU_H) and POU-specific (POU_S) parts and is not the prototype of homeobox proteins because of a unique 21-amino acid insertion in the POU_H part, which interacts with the POU_S to stabilise the interface for efficient transcriptional activity (Cereghini, 1996), (Chi et al., 2002). The HNF1A DBD binds to the GTTAATNATTANC palindromic sequence (Lau et al., 2018). The HNF1A genes encode three isoforms (A, B, and C) that seem to have tissue-specific roles (Bach & Yaniv, 1993), (Harries et al., 2006), of which A is termed the pancreatic isoform, and B and C are both the liver isoforms (Harries et al., 2006).

5.4.1 HNF1A and HNF4A regulatory network

The HNFs family exhibits synergistic relationships in the regulation of tissue development and function. The complex HNF transcriptional regulatory networks have largely been elucidated in rodent models, which was reviewed in (Lau et al., 2018). HNF1A occupies the *HNF4A* promoter region and upregulates its expression as positive feedback (Hansen et al., 2002). Accordingly, in young mice, the downregulation of HNF4A has been associated with the reduced expression of HNF1A (Piccolo et al., 2017). Similarly, together, HNF4A and HNF1A, form a network wherein each controls the expression of the other, as well as multiple liver-specific genes (Hansen et al., 2002), (Kuo et al., 1991). Several studies have demonstrated that HNF1A and HNF4A mutually regulate each other’s expression through DNA-binding-dependent and independent mechanisms (Eeckhoute et al., 2004), (Thomas et al., 2001). The study with HNF4A KD showed substantial reductions in HNF1A and APOB, and the expression of HNF4A, HNF1A, and APOB mRNA are significantly correlated (Taniguchi et al., 2018). In PiZ mouse livers, the downregulation of HNF4A was associated with the reduced expression of CEBPA (CCAAT enhancer-binding protein alpha), HNF1A, and NRF2 at an early age of the mice (Piccolo et al., 2017). Moreover, both HNF1A and HNF4A are directly associated with HCC, with 50% of chronic HBV infection worldwide and HCV infection in low-incidence areas (Petruzziello, 2018). The interaction between the two factors is bidirectional. The expression of HNF4A is inhibited in HCC which exhibits the long-term expression of HBV with a suppression in the level of HNF1A (Wu et al., 2015), (Honda et al., 2016), (Yasumoto et al., 2017), (Park et al., 2020). Furthermore, HCV infection is known to inhibit HNF4A, which correlates with decreased protein levels of microsomal triglyceride transfer protein and HNF1A (Vallianou et al., 2016). These findings suggest that both HNF1A and HNF4A are critical regulators of liver function and, aetiology, and that their dysfunction leads to liver cancer development. However, unlike for HNF4A mutations, the effects of HNF1A mutations on HCC development remain elusive.

5.4.2 HNF1A mutation in metabolic diseases and HCC

Maturity-onset diabetes of the young type 3 (MODY3) is caused by heterozygous mutation in the HNF1A gene. Mutations in HNF1A interfere with HNF1A WT (Wilms' tumor suppressor gene) and the other proteins which act together to control the transcription in liver/ beta cells and may cause diabetes (Herskowitz, 1987). Moreover, MODY3 and hepatocellular adenomas (HCAs) have neen reported to be associated with HNF1A mutations (Willson et al., 2013). The mutations of HNF1A are well established in HCA and are characterised by hepatic steatosis due to increased fatty acid synthesis and the decreased expression of the liver fatty acid-binding protein. The metabolic consequences of biallelic mutations of HNF1α are dysregulated glycolysis, gluconeogenesis, and lipogenesis (Pelletier et al., 2010), (Bluteau et al., 2002). The distribution of the mutation within the protein domain is also an important factor for transcriptional activity and DNA binding. The distribution of the mutations also differs amongst disease types (Jeannot et al., 2010). Diabetes-associated mutations of HNF1A are high at the POU domain. HNF1A S142F and Q146K and R203C/H mutations in the POU domain are known

to disrupt the hydrogen bonds with DNA, while R131Q/W, H143Y, and K158N mutations disrupt phosphate backbone interactions (Chi et al., 2002). MODY3 mutations are predicted to affect POUS-POUH domain interactions, while substitution mutations at these sites diminish transcriptional activity (Chi et al., 2002). These findings expand our understanding that mutation in the POU domain transcription factors regulate critical steps of molecular events and thus function as the molecular basis for diseases associated defects.

HNF1A mutations are also found in HCC (Ding et al., 2018). HNF1A Q511L mutations have shown reduced transactivation activity and impaired nuclear localisation (Ding et al., 2018). HCC mutations are located in the C-terminal transactivation domain, which suggests that reductions in the transcriptional activity of HNF1A due to mutation are associated with HCC (Navalón-García et al., 2006), (Ding et al., 2018). However, a loss-of-function mutation (S247T) of HNF1A in the homeobox domain also affects transcriptional activity which leads to an oncogenic effect in the liver (Liang et al., 2016). As Such, the analysis of somatic mutations located at the functional domain found in HCC may reveal new pathways involved in carcinogenesis and new targets of cancer treatment.

6. Hypotheses

The research hypotheses assume the following:

1.NRF2 (D29A and L30F) mutations found in the functional domain lose their normal structure and promote aberrant transcriptional activity which impacts on hepatocarcinogenesis by upregulating the transcription of the target gene.

2.HNF1A (Y122C, R229Q, V259F) mutations located at the functional domain impact liver carcinogenesis triggered by the loss of function of HNF1A and HNF4A.

3.The loss-of-function of HNF1A induces significant dysregulation in lipid and cholesterol metabolism.

7. Aim of the study

The research aims to accomplish the following:

- ❑ Define the influence of selected NRF2 mutations (D29A, L30F) on malignant hepatocyte (Hepa1-6, Huh7 cell line) transformation by aberrant transcriptional activity.
- ❑ Define the influence of selected HNF1A mutations (Y122C, R229Q, V259F) on malignant transformation of liver carcinoma cells by loss of function and determine the mutational influence in metabolism.

8. Materials and Methods

8.1 Cell culture

Human embryonic kidney cells (HEK293) (obtained from ATCC, CRL-1573, Manassas, VA, USA), Hepa1-6 mouse hepatoma cells (Hepa1-6 cells), and human hepatocyte-derived carcinoma cells (Huh7 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/litre of glucose (Lonza, Basel, Switzerland, 10% fetal bovine serum (FBS) (EURx, Gdansk, Poland), 100 units/mL of penicillin, and 100 units/mL of streptomycin (Lonza). Cells were maintained under 37°C with 5% CO2, and humidified atmosphere in the Heracell 150i (Thermo Fisher Scientific, Waltham, MA, USA) incubator on a T-75 cm2 cell culture flask (Sigma-Aldrich, Saint Louis, MO, USA). In my study (publication 2), I used HEK293 cell because this cell has not express HNF4A and HNF1A endogenously.

8.2 Plasmids and primers

Human NRF2 expression plasmid constructs carrying modifications of the WT gene used in my 1st publication have been published by others and purchased from Addgene (NC16 pCDNA3.1 FLAG NRF2). The pcDNA3-HA-KEAP1 plasmid was a gift from Dr. Masayuki Yamamoto. Mutant variants of human and mouse NRF2 D29A and L30F were created through site-directed mutagenesis by using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The reporter constructs containing 3 antioxidant response element (3xARE) promoters in the pGL vector were kindly given by Dr. Raymond J Deshaies. The reporter construct for the *MMP9* promoter was a gift by Dr. Thomas Iftner. Control plasmids used in my 1st publication are pcCDNA, and FLAG CMV were also used for control experiments. Specific primers were designed for mutagenesis using the QuikChange Primer Design tool (Agilent Technologies, Santa Clara, CA, USA) purchased from Genomed (Warsaw, Poland) and the sequence was listed in the 1st publication. Mutated sequences of the DLG motif were confirmed using Sanger sequencing (Genomed, Warsaw, Poland). In my 2nd publication, to amplify the mouse HNF1A sequence, we isolated the genomic DNA from non-immunogenic mouse hepatoma cells (Hepa1–6) using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). The primers for the selected gene were designed based on the sequence located on the chromosome. Nucleotide sequences for the NotI and KpnI restriction enzymes were added to the forward and reverse primers, respectively. The primers used for cloning the HNF1A plasmid are listed in publication 1. A human HNF1A WT plasmid construct used in this study was procured from Addgene (Teddington, UK). Mutant variants of human HNF1A Y122C and V259F were created through site-directed mutagenesis by using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The HNF4A P1 (−985 to +1 of the P1 *HNF4A* promoter) promoter was cloned into a basic pGL3 vector containing the luciferase gene (Promega, Madison, WI, USA) digested with KpnI and HindIII enzymes (Thermo Fisher Scientific, Waltham, MA, USA) using an In-Fusion® HD Cloning Kit (Takara, Shiga, Japan). The reporter constructs P2 (–371 to –37 from the *HNF4A* transcription start site) and P2-2200 (−2200 to −1 of the P2 *HNF4A* promoter) were purchased from Addgene. CMYC and FLAG CMV vectors were used for control experiments. For mutagenesis, the primers were designed through the QuikChange Primer Design tool (Agilent Technologies) purchased from Genomed and are listed in publication 2. The Y122C, R229Q, and V259F mutated sequences were confirmed using Sanger sequencing (Genomed, Warsaw, Poland).

8.3 Reporter assay

Hepa1-6 and Huh7 cells (2×10^4 cells/well) were plated in 24-well plates, grown to 40–70% confluency. Cells were transiently co-transfected with the reporter and effector plasmids (that are indicated in Figure legends of publication 1) with 100 ng of TK-LUC renilla plasmid as an internal control using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), according to the

manufacturer's protocols. Depending on the experimental design of publication 1, we transfected different plasmids accordingly. For KEAP1 co-transfections, 50 ng of KEAP1 plasmid/well was used. In my publication 2, 5 × 104 HEK293 cells and Huh7 cells were seeded in 24-well plates. After 24 h, the cells were transfected with 100 ng of the mouse and human plasmids indicated in Figure 2A, B of publication 2 using Lipofectamine 3000 (Thermo Fisher Scientific). The cells were transiently co-transfected with 500 ng of an HNF4A promoter-reporter construct containing consensus binding sites upstream of the firefly luciferase and 100 ng of a Renilla luciferase reporter plasmid, as an internal control, using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. Cells were harvested after 48 h and luciferase activity was assayed using a Luciferase Assay Kit (Promega, Madison, WI, USA). Firefly luciferase activity was normalized with Renilla luciferase to control for sample-to-sample variations in transfection efficiency. All reporter assays were repeated independently at least 3 times. Luminescence was measured using a Synergy LX luminometer (Biotek, Winooski, VT, USA).

8.4 Western blotting

The expression of proteins extracted from cells was determined using the Western blotting analysis. A total of 5×10^5 HEK293 cells were plated in 6-well plates and transfected for overexpression with different HNF1A plasmids(WT and mutations) in amounts of 2 µg for 48 h, using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's protocol. After 48h medium was discarded, then cells were washed with phosphate-buffered saline (PBS, Lonza, Basel, Switzerland). After that nuclear protein was extracted from cells. The nuclear protein concentrations from the HNF1A WT and mutant overexpressed cells were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The molecular weight of the protein was estimated with Precision Plus Protein WesternC Standards (Bio-Rad, Hercules, CA, USA). A total of 10 µg of each protein sample was loaded on an SDS-polyacrylamide gel (4% stacking gel; 12% resolving gel). SDS-PAGE was performed in running buffer in the Mini Trans-Blot cell electrophoresis system (Bio-Rad, Hercules, CA, USA). The electrophoresis was run using PowerPac Basic Power Supply (Bio-Rad, Hercules, CA, USA) for 15 min at 120 V and then the voltage was increased to 150 V for about 1h. The protein was transferred from the SDS-PAGE gel to the hydrophobic, 0.45 µm pore size Immobilon-FL PVDF Membrane (Millipore, Burlington, MA, USA) by wet transfer system using the Mini Trans Blot Central Core module (Bio-Rad, Hercules, CA, USA). The electrophoretic transfer was placed at 4 ^{0}C and run at 100 V for 45min in the transfer tank. The membranes were then blocked with 5% skim milk and after that incubated with the antibodies. The blot was incubated overnight at 4°C with mouse monoclonal Anti-Flag antibody (1:5000, Sigma) in 1% skim milk and 0.1% PBST, followed by incubation with HRP-conjugated anti-mouse IgG produced in goats (1:5000, Sigma-Aldrich, Saint Louis, MO, USA) in 1% skim milk and 0.1% PBST for 1 h at room temperature. For the siRNA KD experiment, we used rabbit monoclonal anti-HNF4A (1:1000, Cell Signaling Technology, Danvers, MA, USA) antibody and anti-rabbit IgG produced in goats (1:5000, Sigma-Aldrich). Anti-β-actin (1:1000, Cell Signaling Technology) was used as a loading control. The proteins were visualized using an ECL Western Blotting Analysis System (Amersham, Illinois, CA, USA) and ChemiDoc XRS + System (Bio-Rad, Hercules, CA, USA).

8.5 Immunofluorescence (IFC)

To check the cellular localization of WT and mutant HNF1A we performed IFC staining. 5×10^5 HEK293 cells were plated in 6-well plates and overexpressed with HNF1A WT and mutant plasmids in amounts of 2 µg for 48 h using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's protocol. Following 48h of transfection, the cells were fixed with 4% paraformaldehyde in PBS (Sigma-Aldrich, Saint Louis, MO, USA) for 15 min at room temperature. Next, the cells were rinsed with PBS 0.1% Tween-20 (PBST) and treated with PBS 0.5% Tween-20 (PBST) for 10 min. After that, the cells were blocked in 1% skim milk for 20 min at room temperature. The cells were

washed with PBS 0.1% Tween-20 (PBST) and incubated overnight at 4^0C with mouse monoclonal FLAG-antibody. Then, the cells were washed with PBST and incubated with Alexa546-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. Then cells were washed 3 times with PBST, the cell nuclei were counterstained with 1 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min. The cells were finally washed with PBS and mounted on slides with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). Then cells were observed under a confocal microscope (A1R, Nikon, Tokyo, Japan) equipped with 10x, 20x, 40x and 60x lenses; Nomars- 5 ki's DIC contrast; Hoffman's modulation contrast; 405-, 488-, 561- and 640-nm lasers; a hybrid scanner; and a resonance scanner (Nikon). The workstation was equipped with Nikon's Confocal NIS-Elements package. The confocal images were analyzed using the IMARIS 6.0.1 software (Bitplane AG, Oxford, UK).

8.6 Electrophoretic mobility-shift assay (EMSA)

Cell transfection and nuclear protein samples were extracted as described in western blotting. Oligonucleotides synthesized by Sigma-Aldrich were used for DNA-binding assays. Sequence information is provided in publication 2, Supplementary File 1, Table S2. Generation of double-stranded probes was done by heating equal molar amounts of each of the 5′ to 3′ oligonucleotides with their respective complementary oligonucleotides at 95 °C for 10 min, followed by cooling at room temperature. Next, double-stranded oligonucleotides were labeled with DIG-11-ddUTP using recombinant terminal transferase (20 units/ml) in a final volume of 25 µl, according to the DIG Gel Shift Kit, second generation manufacturer's instructions (Roche Applied Science, Mannheim, Germany). EMSA was performed according to the manufacturer's protocol. DNA-binding reactions were set up using 10 µg of a nuclear extract of either WT or mutant proteins. These proteins were mixed with the above-mentioned DIG-labeled oligonucleotides in a DNA-binding buffer containing 1 µg of poly(dI-dC) and 0.1 µg of poly-l-lysine in a final reaction volume of 20 µl. The DNA-protein complex was separated in a 5% polyacrylamide gel in 0.5X TBE buffer at 100V and then transferred onto a positively charged nylon membrane in 0.5X TBE at 70V for 30 min. The membrane was UV-cross-linked and to bind with DIG-labeled probe was incubated with Anti-Digoxigenin-AP and the labeled probe was detected with CSPD working solution (chemiluminescent EMSA detection) according to the manufacturer's instruction.

8.7 Molecular dynamics (MD) simulations

The structure of the POUh domain (residues 201 to 278, chain B) of HNF1A was derived from the Protein Data Bank; the ID is 1IC8 (Chi et al., 2002). Protein and water molecules within 5 Å of the POUh domain were retained and considered in the initial structure. The N-terminal residue of the protein was capped with an acetyl group to reduce the truncated effect of the POUs domain. Hydrogen atoms were added to the protein and water molecules with the pdb2gmx module of GROMACS (Abraham et al., 2015) under the assumption of the standard protonated state. The simulation system was solvated with TIP3P water molecules (Jorgensen et al., 1983) and neutralized in a dodecahedron box with a minimum distance of 12.0 Å between the protein and the box edges and with 0.15 M concentrations of Na+ and Cl- ions. The AMBER ff14SB parameter set (Maier et al., 2015) and the parameter set previously reported (Joung & Cheatham, 2008) were employed for the force fields of the protein and Na+ and Cl− ions, respectively. The total number of atoms in the box was 29,144. The V259F mutant's structure was modelled using MODDELER (Sali & Blundell, 1993). In the process, residues within 8 Å of the C_β atom of V259 could move to avoid any atomic overlap. The mutant system was also prepared as the WT system above. The total number of atoms in the box was 29,154. All the MD simulations were performed with GROMACS. Ten independent runs were performed as follows: the simulation systems were first subjected to energy minimization with the steepest descent method, followed by the conjugate gradient method. Then, for equilibrating the systems, MD simulations were carried out for 100 ps at 300 K with NVT condition and for another 10 ns at 300 K with NPT condition

using Berendsen's method (Eslami et al., 2010). Finally, for each system, product runs were carried out for 200 ns at 300 K under NPT conditions using the Parrinello–Rahman method (Mencel et al., 2019). The temperature was maintained with Langevin bath (the time constant for coupling was 2 ps) (Goga et al., 2012), and the electrostatic interactions were calculated with the particle mesh Ewald method (Essmann et al., 1995). Non-bonded interactions were cut off at 10 Å, and the bond length including hydrogen atoms was constrained by the LINCS method (Hess et al., 1997) for protein and the SETTLE method (Miyamoto & Kollman, 1992) for the water molecules. The integral time step was set to be 2 fs. For analysis, the last 100 ns trajectories were used. The total MD trajectory for analysis was 1 µs. Residue-wise intra-contact was counted if any of the heavy atoms from a pair of residues was less than 4.5 Å. Then, the differences in contacts between the WT and V259F mutant were calculated by subtracting the contacts of V259F from those of the WT. The last 100 ns of all the 10 trajectories were used for the contact-map calculation. The solvent-accessible surface area was calculated with VMD (Humphrey et al., 1996). The molecular figures were also created with VMD (Humphrey et al., 1996).

8.8 KD by HNF4A siRNA

For KD, a total of 3×10^5 Huh7 cells were plated in 6-well plates and transfected with a 20 nM of either control or HNF4A specific siRNAs by Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and cultured for 48 h in DMEM + 10%FBS medium without antibiotics. The sequences of the siRNAs and primers are listed in publication 2, Supplementary File 1, Table S2. MISSION siRNA Universal Negative Control (SIC-001-s) was obtained from Sigma Genosys (Sigma Genosys Holdings LLC, Texas, USA). After 48 h of transfection, the cells were lysed with T-PER for the extraction of whole-cell protein, and Western blotting was performed as described above.

8.9 RNA isolation, cDNA synthesis and RNA -Sequencing (RNA-Seq) and functional analysis

RNA was extracted from the Huh7 cells after 48 h from cell transfection using RNA extraction kit NucleoSpin RNA (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. RNA concentration was estimated by using spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). OD260/280 and OD260/230 were ≥1.8. To synthesize cDNA, 0.5 µg of total RNA was used as a template, isolated from Huh7 cells using NG dART RT kit, and the reactions were performed according to the manufacturer's protocol (EURx, Gdansk, Poland). PCR was performed with the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Waltham, USA) using the GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA). GAPDH expression was utilized for normalization. RNA-Seq was then carried out via a commercially available service (service ID# F21FTSEUHT1601, BGI, Huada Gene, Wuhan, China). We analyzed the RNA-Seq data for two HNF4A siRNA KD (2 replicates) Huh7 cell samples. The KEGG enrichment pathway and GO bioinformatic analyses were conducted using BGI's Dr. TOM approach, an in-house customized data-mining system of the BGI. The average of 2 controls and the average for the KD (2 siRNA1 and 2 siRNA2) were used to calculate the differential gene expression. The upregulated or downregulated expression of genes was expressed as log2FC, which represents the log-transformed fold change (log2FC = log2[B] − log2[A]).

8.10 Statistical analyses

The data are presented as the means ± standard errors of the means (SEMs) for each group in the experiment. The statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. P values less than 0.05 were considered to indicate statistical significance. The GraphPad PRISM software version 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis

9. Results

To evaluate the prevalence of NRF2 mutations in various human cancers, I searched the International Cancer Genome Consortium (ICGC) database of cancers and found that NRF2 somatic mutations are mostly located at DLG motifs of NRF2 (Publication 1, Table 1) (Zhang et al., 2019). Mutations in the DLG motif found in this study are well conserved among various species (Publication 1, Figure 1A), suggesting a strong effect on protein structure and function. The two somatic mutations D29A or L30F are located in the DLG domain of NRF2 (Fukutomi et al., 2014), which suggestes that mutations at this domain have a significant impact on DNA-binding and protein stability. To examine the effect of the mutations from a structural point of view, NRF2 mutants were modelled based on a crystal structure (PDB code: 3wn7) (Fukutomi et al., 2014) using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. For the D29A mutation, NRF2 loses two hydrogen bonds with KEAP1 R415, which reduces binding affinity by at least 4 kcal/mol. In addition, D29A produces a cavity in the binding site, causing further reductions in binding affinity (Publication 1, Figure 1A, B). NRF2 L30F was not able to fit into the pocket at the KEAP1 surface because the mutation causes a structural clash between NRF2 L30F and KEAP1 R415 and G364 (Publication 1, Figure 1C). Thus, the result suggests that NRF2 mutations in these regions may trigger aberrant NRF2 transcriptional activity due to the loss of DNA binding, and impact liver carcinogenesis. Using Hepa1-6 cells, researchers tested whether NRF2 mutations demonstrate aberrant transcriptional activity in HCC. A luciferase reporter assay showed that the transcriptional activity of mouse and human NRF2 D29A and L30F mutations were increased compared to NRF2 WT when 3xARE reporter was used (Publication 1, Figure 2A, B). Together these data indicate that NRF2 mutations are associated with the gain-of-function activity, which suggests that the DLG domain is important for maintaining proper NRF2 transcriptional activity and that mutations in this domain disrupt proper transcriptional regulation, which can lead to HCC development by increasing the activity of several cancer-related genes. Because KEAP1 is a negative regulator of NRF2, given the importance of the NRF2-KEAP1 system in cancer, the D29A and L30F base substitutions are assumed to potentially affect the DLG motif, and a defective interaction among KEAP1–NRF2 would then result in NRF2 accumulation and thus the increased expression of NRF2 transcriptional targets (Shibata et al., 2008). Following this, NRF2 WT and both D29A and L30F mutations were overexpressed in Hepa1-6 cells in the presence or absence of HA-tagged KEAP1. As expected, the transcriptional activity of NRF2 WT was markedly decreased with KEAP1 co-expression. However, the presence of KEAP1 did not inhibit the transcriptional activity of NRF2 D29A or L30F mutations (Publication 1, Figure 3). This suggests that the loss of KEAP1 function occurs when the NRF2 DLG motif is mutated, and that this causes increased NRF2 transcriptional activity in HCC.

Previous studies have demonstrated that NRF2 levels correlate with the invasiveness and metastatic progress of HCC through the modulation of NRF2 expression (Zhang et al., 2015). Interestingly, NRF2 regulates the expression of MMP9, a protein regulating cell invasion in different cancers, including human HCC (Zhang et al., 2015), (Endo et al., 2018), (Pan et al., 2013). It is hypothesised that NRF2 mutations increase *MMP9* transcription. To test this, both Hepa1-6 and Huh7 cells were transiently transfected with the *MMP9* promoter and human WT and mutant (D29A and L30F) NRF2. Interestingly, the overexpression of the NRF2 D29A and L30F mutants showed a significant enhancement of *MMP9* promoter activity compared to NRF2 WT both in Hepa1-6 and Huh7 cells (Publication 1, Figure 4A, B). These results suggest that NRF2 mutations might contribute to the invasiveness of liver cancer by increasing *MMP9* promoter activity in HCC cells. *BRAF* is one of the most described potential oncogenes. During tumorigenesis, oncogenic *BRAF* leads to the activation of NRF2 (DeNicola et al., 2011). Indeed, the presence of two oncogenic G12D K-Ras and V619E BRAF mutations in murine primary cells increases the expression of NRF2, thereby inducing proliferation and tumorigenesis (DeNicola et al., 2011). Our study examined the synergistic effect of NRF2 and BRAF MTs when overexpressed together, as NRF2 is the downstream target of BRAF-ERK (extracellular signal-regulated kinases) (DeNicola et al., 2011). To determine the synergistic role of NRF2 mutations together with oncogenic BRAF in HCC development, reporter assays were performed using ARE reporter and *MMP9* promoter. When NRF2 D29A and L30F mutations were overexpressed with BRAF V600E mutation, significantly higher transcriptional activity occurred, compared to NRF2 mutations alone (Publication 1, Figure 5A), which suggests that NRF2 mutations achieve more gain-of-function

activity when another oncogenic mutation is occurring. The BRAF mutation has been suggested to be associated with the upregulation of MMP9 expression in several cancers (Frasca et al., 2008), (Guarneri et al., 2017). Next, to examine whether BRAF mutation can regulate NRF2 transcriptional activity by regulating *MMP9* promoter activity, Huh7 cells were transfected with an *MMP9* promoter in the presence of NRF2 WT or mutants (D29A and L30F), with or without BRAF WT or BRAF V600E. Interestingly, BRAF V600E mutation, together with NRF2 (D29A, L30F) mutations, resulted in a marked increase in NRF2 transcriptional activity as assessed through the induction of *MMP9* promoter activity (Publication 1, Figure 5B). It has been reported that MMP9 levels are increased in BRAF V600E - expressing tumours (Salemi et al., 2018). Therefore, these data suggested that BRAF mutation is synergistically involved with NRF2 mutation in the upregulation of NRF2 transcriptional activity through increased *MMP9* transcription.

Next-generation sequencing has helped to identify the low-frequency somatic mutations in HCC and identified HNF1A as a candidate driver gene (Fujimoto, Okada, et al., 2016). HNF1A mutations frequently located in the POU domain of HNF1A and, identified from the ICGC database of liver cancer project, are presented in this study (Publication 2, Figure 1A and Table 1). From an evolutionary perspective, the HNF1A (Y122C, R229Q, and V259F) mutants are strictly conserved among different species (Publication 2, Figure 1B). These data suggest that HNF1A mutations in these regions might exert a strong effect on protein function and hepatocarcinogenesis. HNF1A mutations have been reported to affect DNA binding and reduce transcriptional activity. However, few reports on the functional analysis of disease-associated mutations in HNF1A (Hechtman et al., 2019), (Ding et al., 2018) exist. As such, to determine how the novel mutations affected the transcriptional ability of the HNF1A, a reporter assay was performed using HNF1A-responsive elements containing the *HNF4A* P-1 promoter. Y122C, R229Q human, and Y122C mouse mutations resulted in a decreased transactivation function for HNF1A towards *HNF4A* P1. More importantly, the mouse and human HNF1A V259F mutations completely lost their transcriptional activity in all cases (Publication 2, Figure 2 A, B). These results are consistent with a previous study indicating that MODY3-associated mutants displayed reduced transcriptional activity for their target promoters (Bjørkhaug et al., 2003), (Galán et al., 2011). Next, to investigate the DNA-binding ability of the mutant HNF1A proteins, EMSA (Electrophoretic Mobility-Shift Assay) was performed, and a clear correlation was found between the effects of these mutations on HNF1A transcriptional activation and DNA binding. HNF1A Y122C, R229Q, and V259F mutants showed a significant reduction in DNA binding to the *HNF4A* promoter compared to the WT HNF1A (Publication 2, Figure 2E); however, the WT and mutant HNF1A proteins were expressed equally, as demonstrated by western blot analysis (Publication 2, Figure 2F). Changes in the nuclear localisation of proteins may affect transcriptional activity. Therefore, using IFC (Immunofluorescence) staining, the nuclear localisation ability was analysed. IFC staining revealed that both the WT and mutant HNF1A were localised in the nuclei of HEK293 cells (Publication 2, Figure 2D). Thus, these findings strongly suggest that HNF1A Y122C, R229Q, and V259F mutants have reduced transcriptional activity due to their loss of DNA binding ability to *HNF4A* promoter regions and that these are related to the loss of HNF4A expression and function. Remarkably, the RNA-Seq data obtained from TCGA database of cancer patients showed that *HNF4A* and *HNF1A* mRNA expression is significantly correlated in many cancer types (Publication 2, Supplementary File S1, Figure S3). These results suggest that HNF1A and HNF4A are involved in a cross-regulatory network, and that if a loss-of-function mutation occurs in one, it may lead to the reduced expression of the other. It was thus further investigated whether the structural change occurred when the HNF1A POU domain was mutated.

V259 is located in the POUh domain, related to the DNA-binding region of HNF1A (Publication 2, Figure 3A). Although V259 is not directly involved in protein–DNA interactions, the mutation has been found to reduce binding affinity. To examine the impact of the mutation, MD (Molecular dynamic) simulations with explicit solvent models were conducted for each of the WT and V259F proteins. The root-mean-square fluctuations (RMSFs) showed that the fluctuations of the structures were similar, except for the N-terminal region (Publication 2, Figure 3B). V259F had a significantly higher fluctuation compared to the WT at the N-terminal. The residue-wise contact map, which demonstrates changes in the interaction between the two residues, displayed that V259F lost several key interactions: the hydrophobic interaction of V259–V264 and electrostatic and/or hydrophobic interactions of N237–L258, K205–S256, N237–N257, and R203–S256 (Publication 2, Figure 3C, D). The loss of these

interactions destabilised the hydrophobic packing formed around V259 in the WT. In the WT structure, the V259 was always shielded from the solvent (Publication 2, Figure 3E, F), whereas in the mutant structure, the mutated Phe was often exposed to the solvent. A correlation between the solvent-accessible surface area of the Phe and the fluctuation in the N-terminal region was observed (Publication 2, Figure 3E, F). These large conformational changes in the N-terminal region result in the loss of DNA interactions by R203 and K205, reducing the DNA-binding affinity. Furthermore, this fluctuation affects the arrangement of the POUh and POUs domains, both of which bind to DNA.

Overall, the results suggest that POU domain mutations of HNF1A downregulate *HNF4A* gene expression. As such, to mimic the HNF1A mutation phenotype in transcription networks, siRNA-mediated KD of HNF4A was performed. The HNF4A levels were significantly decreased in Huh7 cells through HNF4A siRNA treatment validated by PCR (Polymerase chain reaction) (Publication 2, Figure 4A). The changes in protein levels were examined by western blot, and HNF4A siRNA was found to markedly reduce HNF4A protein levels as compared with the controls (Huh7 cells transfected with the control siRNA; Publication 2, Figure 4B). HNF4A is a known tumour suppressor, which regulats the transcription of myriad genes (Lau et al., 2018), (Kyithar et al., 2013), (Taniguchi et al., 2018). To further understand the effect of KD on the mechanism underlying HNF4A's tumorigenic function, RNA-Seq analysis was performed to evaluate the genome-wide gene expression profile in HCC cells after HNF4A KD. This analysis showed that 748 genes were differentially expressed in the HNF4A KD cells (Publication 2, Figure 4C). Among these, 311 genes were downregulated, and 437 were upregulated (Publication 2, Figure 4D). Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis revealed that the most overrepresented pathways were the lipid and cholesterol metabolic pathways (Publication 2, Figure 4E). Gene ontology analyses revealed that the genes were mainly involved in biological processes, such as lipid and cholesterol metabolism, and extracellular matrix organisation (Publication 2, Figure 4F). The protein-protein interaction analysis of the downregulated genes revealed that HNF4A KD also caused the downregulation of its target proteins, which are involved in lipid and cholesterol metabolism (Publication 2, Figure 4G).

10. Discussion

NRF2 mutation plays a vital role in cancer progression (Chu et al., 2018), (Kerins & Ooi, 2018), (Zhang et al., 2020). It has been reported that the DLG motif is crucial for maintaining proper NRF2 function and NRF2-ARE mediated gene expression. Genetic alteration on the DLG domain of NRF2 might affect its transcriptional activity (Tong et al., 2007). Furthermore, mutations in the DLG motif change the conserved D29 and L30 residues, which makes NRF2 more vulnerable to structural changes (Shibata et al., 2008). Publication 1 demonstrates the structural model of NRF2mutation with KEAP1 and indicates that the DLG mutations in NRF2 drastically reduce its binding affinity with KEAP1, and cause structural hindrance to the pocket in the interface of NRF2 and KEAP1 (Publication 1, Figure 1B). Previous studies have revealed *NRF2* mutations to be found in cancers associated with the constitutive activation of NRF2 (Guichard et al., 2012), (Kerins & Ooi, 2018), (Orrù et al., 2018), (Fujimoto, Furuta, et al., 2016), (Inami et al., 2011), (Zavattari et al., 2015), and the elevated expression of NRF2 target genes confers advantages in terms of stress resistance and cell proliferation in normal and cancer cells (Ohta et al., 2008). In line with this, to determine the role of HCC associated NRF2, mutation a reporter assay was performed and found a constant increase in NRF2 D29A and L30F mutations transcriptional activity through antioxidant response element 3xARE (Publication 1, Figure 2 A, B). However, KEAP1 could not reduce the transcriptional activity of NRF2 mutations (Publication 1, Figure 3), suggesting a pathogenic mechanism of HCC development via upregulation of several NRF2 target genes.

It has been reported that the transcriptional activation of MMP9 is regulated by NRF2 (Endo et al., 2018). MMP-9 is important for invasion, metastasis, and tumour angiogenesis (Bergers et al., 2000). The invasion process of MMP9 occurs through its positive correlation with NRF2 (Zhang et al., 2015). The results from our study showed that NRF2 D29A and L30F mutations increase the transcriptional activity of the *MMP9* promoter through NRF2 induction in carcinoma cells (Publication 1, Figure 4 A, B), which suggests that mutations are linked with the development of HCC via increasing cell invasiveness and cells with mutations developing malignant phenotypes. A aberrant NRF2 expression that increases *MMP9* promoter activity in HCC cells can thus be considered a critical target for the development of novel therapeutics. Oncogenic pathways have been reported to increase NRF2 transcription via the activation of the B-Raf-MEK-ERK and to support its pro-tumorigenic effects (DeNicola et al., 2011). In line with this assumption, our study provides evidence for the first time that the transcriptional activity of NRF2 D29A and L30F mutations was increased with the overexpression of oncogenic BRAF mutation (Publication 1, Figure 5 A, B). BRAF mutation altered the tumour microenvironment by regulating the MAPK pathway (Zipper & Mulcahy, 2003), this enhances ERK phosphorylation, which leads to the overexpression of several genes in the nucleus involved in tumour development, including *MMP9* (Salemi et al., 2018), (Zipper & Mulcahy, 2003). Notably, high levels of MMP9 together with- BRAF mutation are associated with poor overall survival in melanoma patients (Salemi et al., 2018), and the activation of NRF2 through this pathway might be critical for tumour cell proliferation. These results have revealed a critical role played by an NRF2-BRAF-MMP9 signal that could serve as a basis for HCC progression when genes are dysregulated. However, the high activity of NRF2 mutations in HCC together with BRAF mutation warrants further investigation to develop the potential diagnostic, prognostic, and therapeutic utility of this pathway in HCC.

Previously, HNF1A mutations were identified in diabetes, and their functional effect has already been validated (Bjørkhaug et al., 2000), (Plengvidhya et al., 2019), (Beysel et al., 2019); however few studies have been performed with HNF1A mutations, which are associated with the development and progression of HCC (Ding et al., 2018). Interestingly, HNF1A was one of the commonly mutated genes found in HCC, according to the ICGC database (https://dcc.icgc.org/), and most of the mutations are located at the POU domain of HNF1A (Publication 2, Table 1). The HNF family shares common features, such as DNA binding and transactivation capabilities, which are responsible for its functional diversity (Thomas et al., 2001), (Balamurugan et al., 2016); HNF family gene mutations mostly occur in the functional domain of the protein and inhibit the protein's activity by affecting its DNA-binding affinity and protein conformation (Taniguchi et al., 2018), (P et al., 2017). Previously reported POUh domain R271W and S247T mutations of HNF1A impair HNF1A's transcriptional activity to

transactivate the HNF4A promoter (Galán et al., 2011),. These results are consistent with the data from my study (Publication 2), in which I found the impaired transcriptional activity of HNF1A Y122C, R229Q, and V259F mutants in the regulation of HNF4A promoter activity (Publication 2, Figure 2 A, B, C). Moreover, the HNF1A transactivation domain mutation Q511L has been reported to diminish the function of HNF1A and it disturbs *HNF4A* promoter activity, and consequently inhibits the proliferation, migration, and invasion of HCC cells (Ding et al., 2018). My results thus suggest that the reduction of HNF4A promoter activity caused by HNF1A POU-domain mutations may play a role in HCC development by downregulating its target gene. While our study emphasises the importance of POU-domain mutations of HNF1A, further functional studies are needed to verify the mutations found in different cancers. Likewise, as HNF1A mutations are commonly found in MODY patients, it is clinically important to verify the risk of liver cancer development in this group. In mice, the hepatocyte-specific deletion of HNF1A leads to the spontaneous development of HCC due to fatty liver without cirrhosis and NASH (Ni et al., 2017). Similarly, the KO/KD (Knockout/Knockdown) of the major HNF1A target gene HNF4A is known to play a role in liver oncogenesis or HCC (Cai et al., 2017), (Walesky et al., 2013), which suggests that both HNF1A and HNF4A account for maintaining liver homeostasis, and that the disruption of their function may lead to liver pathologies. In Publication 2, it was observed that HNF1A Y122C, R229Q, and V259F mutations significantly reduced the DNA-binding capacity of HNF1A for the *HNF4A* promoter (Publication 2, Figure 2E). This study, together with others, suggests that HNF1A and HNF4A are involved in a regulatory network (Taniguchi et al., 2018), (Harries et al., 2009), (Morimoto et al., 2017); as such, pathogenic mutations in either the HNF1A or HNF4A gene may increase the risk of HCC by reducing the expression of the other. A few studies have revealed that the HNF1A–HNF4A axis is an important pathway for the control of liver homeostasis and that its disruption can cause dysregulated liver function.

MD simulations allowed us to elucidate the dynamic nature of the protein–DNA interaction (Publication 2, Figure 3D). A higher RMSF is associated with reduced protein stability (P et al., 2017), which is consistent with our observation that the V259F mutant complex exhibited a greater fluctuation pattern (Publication 2, Figure 3C). Those proteins with arginine residues on their surfaces demonstrate greatly increased stability (Strub et al., 2004). conversely, in my study, the rearrangement of arginine and lysine residues results in reduced stability and negatively affects the protein function. The complete loss of DNA binding for V259F suggests that valine is an essential amino acid critical for DNA interaction and binding affinity. As such, in this study, the large conformational changes in the N-terminal region, but not the change in protein stability, resulted in the loss of DNA interactions by rearranging R203 and K205, thus reducing the DNA-binding affinity.

Publication 2 found that loss-of-function mutations to the HNF1A POU domain cause a reduction in HNF4A gene expression. However, the molecular mechanism through which the loss - of - function may cause disrupted gene expression at the molecular level remains unclear. To determine the molecular mechanisms of this function, we performed a global gene expression analysis in the condition of HNF4A KD. The top seven downregulated genes were HPR, PKLR, PLAU, SOAT2, IYD, OTC, and ASGR1. OTC is known as prognostic biomarker in HCC, and its deficiency in the liver leads to ammonia deposition, which causes chronic liver damage (Wilson et al., 2012). Moreover, heterologous OTC-KO mice developed liver fibrosis (Wang et al., 2017). Therefore, low OTC expression may enable tumour cells to enhance ammonia accumulation, which represents a loss of function of OTC in tumour-specific metabolism. ASGR1 overexpression inhibits hepatoma cell migration and invasion by interacting with LASS2 (Gu et al., 2016). Here, we found that the expression of ASGR1 was downregulated in HNF4A KD cells, which suggests that HNF4A positively regulates ASGR1 expression in HCC cells. Our results are thus consistent with the previous report and suggest the tumour suppressor role of ASGR1 in HCC (Peters et al., 2016), the IYD overexpression inhibited Huh7 cell growth by inhibiting glycolysis in HCC (C. Lu et al., 2020), the IYD downregulation found in my study is considered a key driver in HCC malignancy. However, the contribution of IYD in relation to HNF family genes to tumorigenesis in the liver has yet not been studied and warrants further examination. HPR, PKLR, and PLAU have been reported to be overexpressed in breast cancers and oesophageal squamous cell carcinoma (Kuhajda et al., 1989), (Y. Yang et al., 2019), (Fang et al., 2021). However,

in contrast, this study showed the downregulation of those genes. It is plausible that these genes might have tissue-specific roles, and that their downregulation may promote the transition from liver damage to hepatocarcinogenesis, but further studies are required to validate this hypothesis. In addition, several downregulated genes in the HNF4A gene network are involved in lipid and cholesterol metabolism:- GATA4, APOC3, APOA1, SOAT2, and FOXO1 were found to be downregulated in Huh7 HNF4A KD cells, known as cholesterol and lipid metabolism-related genes (Winkler et al., 2021), (Marinho et al., 2018). Notably, the hepatocyte-specific deletion of Gata4 mice developed enlarged livers with a proliferative phenotype (Enane et al., 2017), which suggests a role in liver cancer development. The overall findings in Publication 2, suggest that HNF4A is one of the master regulators of lipid and cholesterol homeostasis, and that the disruption of HNF1A protein function caused by mutations may trigger liver cancer development and progression due to the disruption of lipid and cholesterol metabolism as well as key liver functions such as ammonia and glycoprotein homeostasis. Further in vitro and in vivo studies are required to evaluate the mutational effect of HNF1A on HCC development.

11. Conclusions

1. NRF2 D29A and L30F mutations induce the aberrant transcriptional activity of NRF2-ARE, and MMP9 promoter in Hepa1-6 and Huh7 cells may induce the development of liver cancer.
2. BRAF V600E mutation induces NRF2 mutations transcriptional activity through increased MMP9 transcription, which suggests that NRF2-BRAF-MMP9 signalling may induce cell proliferation and invasion in liver tumours.
3. HNF1A Y122C, R229Q, and V259F mutations can be risk factors for triggering HCC through the alterations of multiple gene expressions via loss-of-function activity and DNA binding.
4. HNF1A mutations regulate HNF4A promoter, and vice-versa.
5. The KD of HNF4A downregulates HNF1A and other target genes related to lipid and cholesterol metabolism.
6. Therapy based on the inhibition of the NRF2-BRAF-MMP9 signalling pathway might be applied in patients with liver cancer carrying NRF2-BRAF mutations. The forced overexpression of HNF1A/4A in the mutational background of cancer might also provide therapeutic efficiency.

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13. Attachment

13.1 Publications constituting doctoral dissertation

13.2 Statement of authors

International Journal of
Molecular Sciences

Article

NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines

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Abstract: Geographically, East Asia had the highest liver cancer burden in 2017. Besides this, liver cancer-related deaths were high in Japan, accounting for 3.90% of total deaths. The development of liver cancer is influenced by several factors, and genetic alteration is one of the critical factors among them. Therefore, the detailed mechanism driving the oncogenic transformation of liver cells needs to be elucidated. Recently, many researchers have focused on investigating the liver cancer genome and identified somatic mutations (MTs) of several transcription factors. In this line, next-generation sequencing of the cancer genome identified that oxidative stress-related transcription factor NRF2 (NFE2L2) is mutated in different cancers, including hepatocellular carcinoma (HCC). Here, we demonstrated that NRF2 DLG motif mutations (NRF2 D29A and L30F), found in Japanese liver cancer patients, upregulate the transcriptional activity of NRF2 in HCC cell lines. Moreover, the transcriptional activity of NRF2 mutations is not suppressed by KEAP1, presumably because NRF2 MTs disturb proper NRF2-KEAP1 binding and block KEAP1-mediated degradation of NRF2. Additionally, we showed that both MTs upregulate the transcriptional activity of NRF2 on the *MMP9* promoter in Hepa1-6 and Huh7 cells, suggesting that MT derived gain-of-function of NRF2 may be important for liver tumor progression. We also found that ectopic overexpression of oncogenic BRAF WT and V600E increases the transcriptional activity of NRF2 WT on both the 3xARE reporter and *MMP9* promoter. Interestingly, NRF2 D29A and L30F MTs with oncogenic BRAF V600E MT synergistically upregulate the transcription activity of NRF2 on the 3xARE reporter and *MMP9* promoter in Hepa1-6 and Huh7 cells. In summary, our findings suggest that MTs in NRF2 have pathogenic effects, and that NRF2 MTs together with oncogenic BRAF V600E MT synergistically cause more aberrant transcriptional activity. The high activity of NRF2 MTs in HCC with BRAF MT warrants further exploration of the potential diagnostic, prognostic, and therapeutic utility of this pathway in HCC.

Keywords: NRF2; KEAP1; somatic mutation; transcriptional activity; BRAF; MMP9; HCC

1. Introduction

More than 50% of the global liver cancer burden is located in East Asia. After China (51.03%), liver cancer-related deaths are highest in India and Japan, accounting for 4.33%

and 3.90%, respectively, of the global deaths in 2017 [1]. Epidemiologically, alcohol consumption and hepatitis virus (HBV, HCV) infection, as well as the occurrence of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), have been reported as risk factors for hepatocellular carcinoma (HCC) [2–8]. Furthermore, the primary etiological factor for liver cancer in Japan is HCV infection [1]. HCV infection causes oxidative stress and activates nuclear factor erythroid-2–related factor 2 (NRF2) [9]. NRF2 is an oxidative stress-related transcription factor reported as a potential prognostic marker for HCC development and progression [10,11]. However, the detailed understanding of how NRF2 reacts as oncogene in liver cells remains unknown. Recent findings suggest that NRF2 promotes cancers because of somatic mutations (MTs) that cause aberrant NRF2 transcriptional activity [12]. Whole exome sequence analysis identified that around 6.4% of MTs found in tissues of HCC-affected patients are present in the NRF2 gene. Interestingly, these MTs are located within the *Asp*-Leu-Gly (DLG) and *Glu-Thr-Gly-Glu* (ETGE) motifs (KEAP1 binding elements) of NRF2, which are important for binding with its negative regulator, KEAP1 [13]. A search of the mutation database revealed that somatic MTs encompassing the NRF2-DLG motif cover a greater region than MTs in the ETGE motif [14]. Moreover, NRF2 MTs are an early event in rats fed with choline-devoid, methionine-deficient (CMD) diet-promoted preneoplastic hepatic nodules, and all MTs are confined within the DLG (74%) or ETGE (26%) motif of exon 2 of the NRF2 gene [15]. A study of diethylnitrosamine (DEN) induced HCC in mice revealed that MTs in the DLG motif of NRF2 are a crucial driver for HCC [10]. Besides these, it was reported that V32E represents the most frequent DLG MT (weak bond), while T80A is the most frequent ETGE MT (strong bond) [14]. This unique nature of KEAP1 binding with DLG motif enables the prompt response of NRF2 to oxidative and electrophilic stress [14]. MTs in this domain specifically alter the amino acids that affect the interaction between NRF2 and KEAP1. Moreover, it has been reported that the DLG motif is a weaker KEAP1-binding site than the ETGE motif. This makes the DLG region more vulnerable to structural changes, and any MTs in this motif are predicted to have a great influence on tumor growth [14,16]; however, the functional importance of these DLG MTs in liver cancer cells is not well understood. Several lines of evidence indicate that overexpression of NRF2 is highly related to cancer development [10,11,15]. The KO of Nrf2 using mice suggested that suppression of aberrant NRF2 activity could reduce the tumor burden [10]. Interestingly, loss of function MTs in KEAP1 overactivate NRF2 and provide benefits for lung cancer cell growth [17]. It is possible that NRF2 might interact with other signaling pathways, which control the tumor survival signal as a result of NRF2 overactivation.

A recent study reported that the matrix metalloproteinase (MMP) family gene MMP9 is one of the targets of NRF2; the MMP9 gene contains two putative antioxidant response elements (ARE), which are known target sequences for NRF2, in its promoter region [18]. Interestingly, NRF2 promotes invasion in human HCC partly through regulating the expression of MMP9 [11]. An in vitro study with HepG2 cells showed that upregulation of the NRF2 pathway stimulates target gene expression, including MMP9, which increases the invasiveness of HCC cells [11]. On the other hand, BRAF (v-raf murine sarcoma viral oncogene homolog B1) is described as a potential oncogene that plays an important role in NRF2 activation [19]. It has been reported that BRAF V600E MT is responsible for melanoma progression through activation of the downstream MEK/ERK pathway [20]. BRAF phosphorylates ERK via MEK in cancer cells, and phospho-ERK phosphorylates its downstream targets, which include NRF2 [21–23]. During tumorigenesis, oncogenic BRAF has been reported to augment NRF2 activity [21]. Cancer cells with NRF2 MTs exhibit high levels of transcriptional activity and maintain malignant tumor growth [24]. Moreover, higher levels of MMP-9 and BRAF V600E MT are associated with lower progression-free survival and overall survival [25]. However, no conclusive findings on the occurrence and transcriptional activity of oncogenic MTs in the coding region regulating the tumor progression process have yet been published. On the basis of these observations, we hypothesized that MTs in the coding region of NRF2 might cause aberrant transcriptional

activity and have some effect on MMP9 transactivation when BRAF MT is also present in liver cancer cells.

Our study successfully revealed that NRF2 gene MTs found in HCC increase the transcriptional activity of NRF2. MTs cause NRF2 to lose its normal structure and hamper a NRF2-KEAP1 interaction. We also found that NRF2 MTs induce the transcriptional activity of the *MMP9* promoter, thereby driving increased *MMP9* expression that is linked to tumor invasion [11,26]. Furthermore, NRF2 D29A and L30F MTs together with BRAF V600E MT play crucial roles in hepatic transcriptional regulation.

2. Results

2.1. NRF2 Mutations Are Mostly Located in the DLG Motif of NRF2

To evaluate the prevalence of NRF2 MTs in different human cancers, we searched the International Cancer Genome Consortium (ICGC) database of different cancers and found that NRF2 somatic MTs in different cancers were mostly located at DLG motifs of NRF2 (Table 1) [27]. The whole-genome sequencing analysis of Japanese liver cancer patients identified two somatic MTs found in DLG domains. From an evolutionary perspective, MTs in the DLG motif found in this study are well conserved among various species (Figure 1A). The highly conserved elements among the analyzed species (human, mouse, bovine, and zebrafish) are highlighted in red and are 100% conserved throughout the different species and the highly conserved DLG domain is indicated by green lines. The MTs in such evolutionary conserved elements suggest a strong effect on protein structure and function. Since D29 and L30 are located in the functional domain of NRF2 [14], it is expected that MTs at these sites have a significant impact on DNA-binding and protein stability. To examine the effect of the MTs from a structural point of view, we modelled the mutants based on a crystal structure (PDB code: 3wn7) [14] using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. For the D29A MT, NRF2 loses two hydrogen bonds with R415, reducing the binding affinity by at least 4 kcal/mol. In addition, D29A produces a cavity in the binding site, causing further reductions in binding affinity (Figure 1A,B). NRF2 with a L30F MT will not be able to fit into the pocket at the KEAP1 surface because the MT causes a structural clash between L30F of NRF2 and R415 and G364 of Keap1 (Figure 1C). Thus, these data suggest that NRF2 MTs in these regions may trigger abberant NRF2 transcriptional activity and impact liver carcinogenesis. However, the functional importance of these MTs in HCC remains to be studied.

Table 1. Novel human NRF2 DLG MTs identified in different cancers and in ICGC database.

MT ID	DNA Change	Type	Amino Acid Change	Project	Tumor Type	Tumor Subtype	Donors Affected
MU1324215	chr2:g.178098960C>G	single base substitution	D29H	LUSC-US	Lung cancer	Squamous cell carcinoma	5/485 (1.03%)
				CESC-US	Cervical cancer	Cervical squamous cell carcinoma	2/289 (0.69%)
				HNSC-US	Head and neck cancer	Squamous cell carcinoma	3/508 (0.59%)
				LUSC-KR	Lung cancer	Adenocarcinoma, squamous cell carcinoma	1/170 (0.59%)
				BLCA-US	Bladder cancer	Invasive urothelial bladder cancer	2/411 (0.49%)
				LICA-FR	Liver cancer	Hepatocellular carcinoma (secondary to alcohol and adiposity)	1/252 (0.40%)

Table 1. *Cont.*

MT ID	DNA Change	Type	Amino Acid Change	Project	Tumor Type	Tumor Subtype	Donors Affected
				ESCA-CN	Esophageal cancer	Squamous carcinoma	1/332 (0.30%)
				UCEC-US	Endometrial cancer	Uterine corpus endometrial carcinoma	1/531 (0.19%)
				LUAD-US	Lung cancer	Adenocarcinoma	1/516 (0.19%)
MU1327674	chr2:g.178098960C>T	single base substitution	D29N	LUSC-US	Lung cancer	Squamous cell carcinoma	5/485 (1.03%)
				LUSC-KR	Lung cancer	Adenocarcinoma, squamous cell carcinoma	1/170 (0.59%)
				LICA-CN	Liver cancer	Hepatocellular carcinoma HBV-associated	1/402 (0.25%)
				LINC-JP	Liver cancer	Hepatocellular carcinoma (virus associated)	1/394 (0.25%)
MU1316143	chr2:g.178098960C>A	single base substitution	D29Y	LUSC-US	Lung cancer	Squamous cell carcinoma	2/485 (0.41%)
				CESC-US	Cervical cancer	Cervical squamous cell carcinoma	1/289 (0.35%)
				BLCA-US	Bladder cancer	Invasive urothelial bladder cancer	1/411 (0.24%)
				HNSC-US	Head and Neck cancer	Squamous cell carcinoma	1/508 (0.20%)
MU871836	chr2:g.178098959T>C	single base substitution	D29G	LICA-FR	Liver cancer	Hepatocellular carcinoma (secondary to alcohol and adiposity)	2/252 (0.79%)
				LUSC-KR	Lung cancer	Adenocarcinoma, squamous cell carcinoma	1/170 (0.59%)
				ORCA-IN	Oral cancer	Gingivobuccal	1/178 (0.56%)
				LINC-JP	Liver cancer	Hepatocellular carcinoma (virus associated)	2/394 (0.51%)
				LUSC-US	Lung cancer	Squamous cell carcinoma	2/485 (0.41%)
				LICA-CN	Liver cancer	Hepatocellular carcinoma HBV-associated	1/402 (0.25%)
				LUAD-US	Lung cancer	Adenocarcinoma	1/516 (0.19%)

Table 1. *Cont.*

MT ID	DNA Change	Type	Amino Acid Change	Project	Tumor Type	Tumor Subtype	Donors Affected
MU1330977	chr2:g.178098957G>A	single base substitution	L30F	LUSC-US	Lung cancer	Squamous cell carcinoma	4/485 (0.82%)
				LIRI-JP	Liver cancer	Hepatocellular carcinoma (virus associated)	1/258 (0.39%)
				PACA-CA	Pancreatic cancer	Ductal adeno-carcinoma	1/268 (0.37%)
				HNSC-US	Head and neck cancer	Squamous cell carcinoma	1/508 (0.20%)
MU1292484	chr2:g.178098953C>G	single base substitution	G31A	LUSC-US	Lung cancer	Squamous cell carcinoma	5/485 (1.03%)
				ESCA-CN	Esophageal cancer	Squamous carcinoma	1/332 (0.30%)
				BLCA-US	Bladder cancer	Invasive urothelial bladder cancer	1/411 (0.24%)
MU866686	chr2:g.178098953C>T	single base substitution	G31E	LUSC-KR	Lung cancer	Adenocarcinoma, squamous cell carcinoma	2/170 (1.18%)
				LINC-JP	Liver cancer	Hepatocellular carcinoma (Virus associated)	2/394 (0.51%)
MU83818151	chr2:g.178098954C>T	single base substitution	G31R	LICA-FR	Liver cancer	Hepatocellular carcinoma (secondary to alcohol and adiposity)	1/252 (0.40%)
				BLCA-US	Bladder cancer	Invasive urothelial bladder cancer	1/411 (0.24%)
				LUAD-US	Lung cancer	Adenocarcinoma	1/516 (0.19%)
MU623518	chr2:g.178098956A>T	single base substitution	L30H	KIRC-US	Renal cancer	Clear cell carcinoma	1/361 (0.28%)
				HNSC-US	Head and neck cancer	Squamous cell carcinoma	1/508 (0.20%)
MU130685128	chr2:g.178098953C>A	single base substitution	G31V	LUSC-US	Lung cancer	Squamous cell carcinoma	1/485 (0.21%)
MU830878	chr2:g.178098956A>C	single base substitution	L30R	LINC-JP	Liver cancer	Hepatocellular carcinoma (virus associated)	3/394 (0.76%)
				KIRP-US	Renal cancer	Papillary carcinoma	1/278 (0.36%)
MU131168581	chr2:g.178098956A>G	single base substitution	L30P	HNSC-US	Head and neck cancer	Squamous cell carcinoma	1/508 (0.20%)
MU29615597	chr2:g.178098959T>G	single base substitution	D29A	LIRI-JP	Liver cancer	Hepatocellular carcinoma (virus associated)	1/258 (0.39%)

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           ..........10 ........20 ........30 ........40 ........50
human      MMDLELPPPG LPSQQDMDLI DILWRQDIDL GVSREVFDFS QRRKEYELEK
mouse      MMDLELPPPG LQSQQDMDLI DILWRQDIDL GVSREVFDFS QRQKDYELEK
bovine     MMDLELPPPG LPSQQDMDLI DILWRQDIDL GVSREVFDFS QRQKEHELEK
zebra_fish MMEIEMSKM- QPSQQDMDLI DILWRQDVDL GAGREVFDFS YRQKEVELRR
Consistency **88*86665 66******** ********9** *77******* 6*7*84**78
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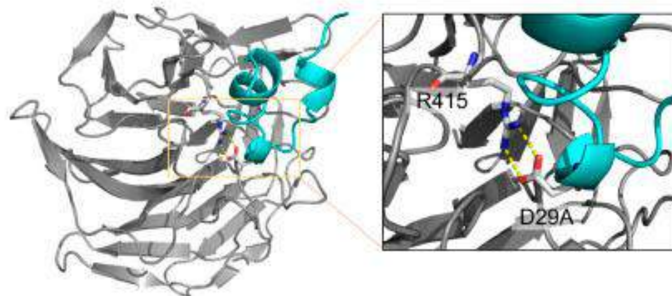
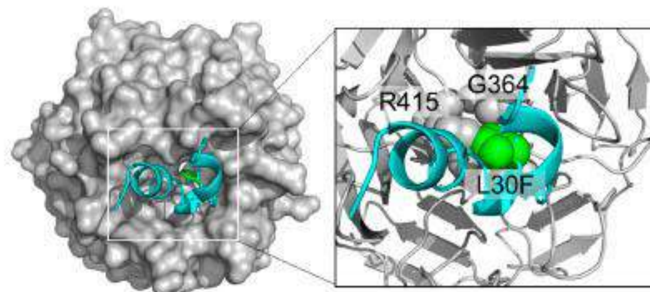
A



B



C

Figure 1. Evolutionally conserved NRF2 DLG domain and structural simulation of KEAP1/NRF2 MTs complex. (**A**) The alignment of the human, mouse, bovine, and zebrafish Nrf2 amino acid sequence. The red color box shows highly conserved (100%) elements among the species. DLG domain is indicated by green lines. (**B**) KEAP1 (gray) and NRF2 DLG (cyan) are shown by cartoon model. Hydrogen bonds between R415 of KEAP1 and D29 of NRF2 are shown by yellow dotted lines. (**C**) KEAP1 and NRF2 DLG are represented by surface (colored in gray) and cartoon (colored in cyan) models, respectively. In the enlarged view, G364 and R415 of KEAP1 are represented by gray spheres, whereas L30F of NRF2 is represented by a green sphere. All images were drawn using PyMOL.

2.2. *NRF2 DLG Mutations Have a Gain-of-Function Activity*

In our study, mouse Hepa1-6 cells were used to test whether NRF2 MTs have aberrant transcriptional activity in HCC. The possibility was explored by transfecting Hepa1-6 cells with mouse WT NRF2 or NRF2 MTs (D29A and L30F) in the presence of a 3xARE reporter. Luciferase reporter assay showed that the transcriptional activity of NRF2 D29A and L30F MTs was increased compared to NRF2 WT (Figure 2A). Additionally, we compared the

transcriptional activities of human NRF2 MTs (D29A and L30F) to that of human WT NRF2. We found that although human NRF2 WT can increase ARE-luciferase activity, D29A and L30F MTs were more than two-fold active when compared to the WT ($p < 0.05$). Together these data indicate that NRF2 MTs are associated with gain-of-function activity (Figure 2B). This suggests that the DLG domain is important to maintain proper NRF2 transcriptional activity, and MTs in this domain disrupt proper transcriptional regulation, which can lead to HCC development by increasing the activity of several cancer-related genes.

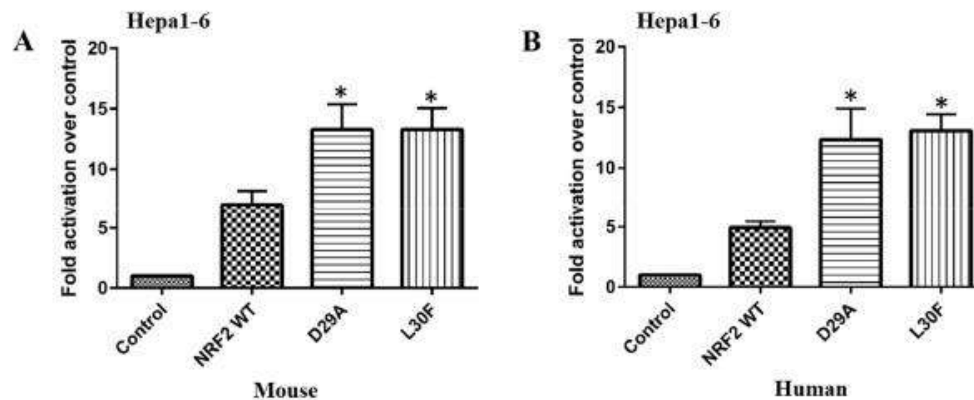


Figure 2. NRF2 MTs increase transactivation potential on its target promoter regions. (**A**). The transcriptional activity of mouse NRF2 WT and MTs. (**B**). The transcriptional activity of the human NRF2 WT and MTs in Hepa1-6 cells. In both experiments, cells were cotransfected with 3xARE luciferase reporters along with either an empty expression vector (serving as a control) or expression vectors (50 ng) for the indicated NRF2 in 24-well culture plates. The bars indicate fold activation of NRF2 WT and MTs (vs. control) on a NRF2 target promoter. Promoter activity is reported as fold activation over control. Data represent the mean ± SEM of 9 (**A**) and 5 (**B**) independent experiments (*, $p < 0.05$).

2.3. *KEAP1 Expression Fails to Reduce the Transcriptional Activity of NRF2 MTs*

Given the importance of the NRF2-KEAP1 system in cancer, we evaluated the effect of KEAP1 on NRF2 DLG MTs in HCC. The D29A and L30F base substitutions might affect the DLG motif within the Neh2 domain by altering the sequence to ALG/DFG. A defective interaction among KEAP1–NRF2 would then result in NRF2 accumulation and thus increased expression of NRF2 transcriptional targets [12]. Because KEAP1 is a negative regulator of NRF2, we proceeded to analyze the transcriptional activity of NRF2 MTs in the presence of KEAP1. The transcriptional activity of NRF2 was determined by analyzing the activity of 3xARE-luciferase reporter plasmids. NRF2 WT and both D29A and L30F MTs were overexpressed in Hepa1-6 cells in the presence or absence of HA-tagged KEAP1. D29A and L30F NRF2 MT proteins had higher transcriptional activity than NRF2 WT (Figure 3). As expected, the transcriptional activity of NRF2 WT was markedly decreased with KEAP1 co-expression. Interestingly, the presence of KEAP1 did not inhibit the transcriptional activity of NRF2 D29A and L30F MTs (Figure 3). This suggests that loss of KEAP1 function occurs when NRF2 D29A and L30F are mutated, and this translates to increased NRF2 transcriptional activity in HCC.

2.4. *NRF2 Mutations Increase the Transcriptional Activity of the MMP9 Promoter*

Previous studies demonstrated that NRF2 levels correlate with invasiveness and metastatic progress of HCC through modulation of NRF2 expression [11]. Interestingly, NRF2 regulates the expression of MMP9, a protein regulating cell invasion in different cancers, including human HCC [11,18,28]. Therefore, we hypothesized that NRF2 MTs

increase MMP9 transcription. To test this, we transiently cotransfected Hepa1-6 and Huh7 cells with the MMP9 promoter and human WT and MTs (D29A and L30F) NRF2. As predicted, NRF2 WT overexpression increased MMP9 promoter activity in both Hepa1-6 and Huh7 cells, which indicates that MMP9 expression is regulated by NRF2 in HCC. Interestingly, overexpression of NRF2 D29A and L30F MTs resulted in a significant enhancement of MMP9 promoter activity compared to NRF2 WT both in Hepa1-6 and Huh7 cells (Figure 4A,B). Taken together, these results suggest that NRF2 MTs increase MMP9 promoter activity in HCC cells, which might contribute to the invasiveness of liver cancer.

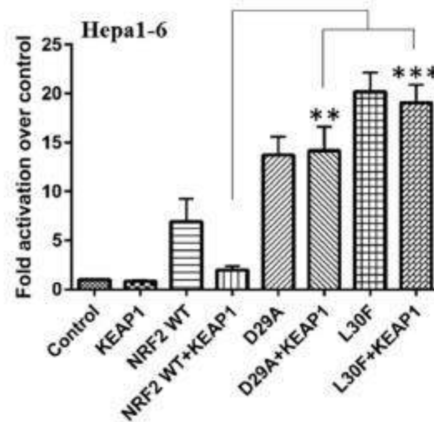


Figure 3. The effect of KEAP1 on NRF2 WT and MTs transcriptional activity in Hepa1-6 cells. The co-expression of KEAP1 inhibited the transcription activity of NRF2 WT but not of the D29A and L30F MTs, indicating that MTs block KEAP1-mediated regulation. The bars indicate fold activation of NRF2 WT and MTs (vs. control) on a NRF2 target promoter. The data represent the mean ± SEM of 4 independent experiments (**, $p < 0.01$; ***, $p < 0.001$).

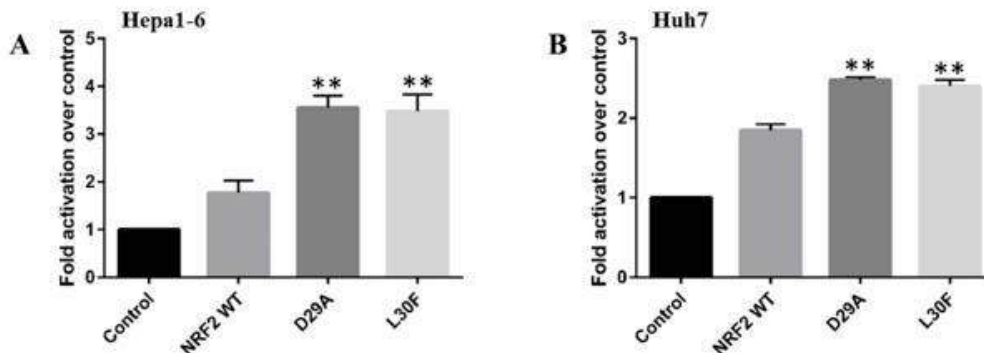


Figure 4. The ability of the NRF2 WT and MTs to transactivate *MMP9* promoters in Hepa1-6 (**A**) and Huh7 (**B**) cells. Cells were cotransfected with a *MMP9* luciferase reporter (250 ng) along with either an empty expression vector (serving as a control) or expression vectors (50 ng) for the indicated NRF2 in 24-well culture plates. Data represent the mean ± SEM of 6 (**A**) and 3 (**B**) independent experiments (**, $p < 0.01$).

2.5. Both NRF2 and BRAF Mutations Increase the Transcriptional Activity of Target Promoters

BRAF is one of the most described potential oncogenes. During tumorigenesis, oncogenic *BRAF* MT leads to activation of NRF2 [21]. Indeed, the presence of two oncogenic G12D K-Ras and V619E B-Raf MTs in murine primary cells increases the expression of NRF2, thereby inducing proliferation and tumorigenesis [21]. Our study examined the synergistic effect of NRF2 and BRAF MTs when overexpressed together, as NRF2 is the downstream target of BRAF-ERK [21]. To determine the synergistic role of NRF2 MTs together with oncogenic BRAF in HCC development, we overexpressed a 3xARE luciferase construct (which is sensitive to NRF2-mediated transactivation) along with NRF2 WT and MTs (D29A and L30F) and BRAF WT and BRAF V600E MT in Hepa1-6 cells. NRF2 D29A and L30F MTs showed higher transcriptional activity compared to WT NRF2 (Figure 5A). Likewise, when NRF2 MTs were overexpressed with BRAF WT, it showed higher transcriptional activity compared to NRF2 MTs alone (Figure 5A). Furthermore, overexpression of BRAF V600E MT yielded higher levels of NRF2 transcriptional activity compared to BRAF WT. Interestingly, there was a marked increase in NRF2 transcriptional activity when both NRF2 (D29A and L30F) and BRAF V600E MTs were overexpressed (Figure 5A), suggesting that NRF2 MTs achieve more gain-of-function activity when there is another oncogenic MT present.

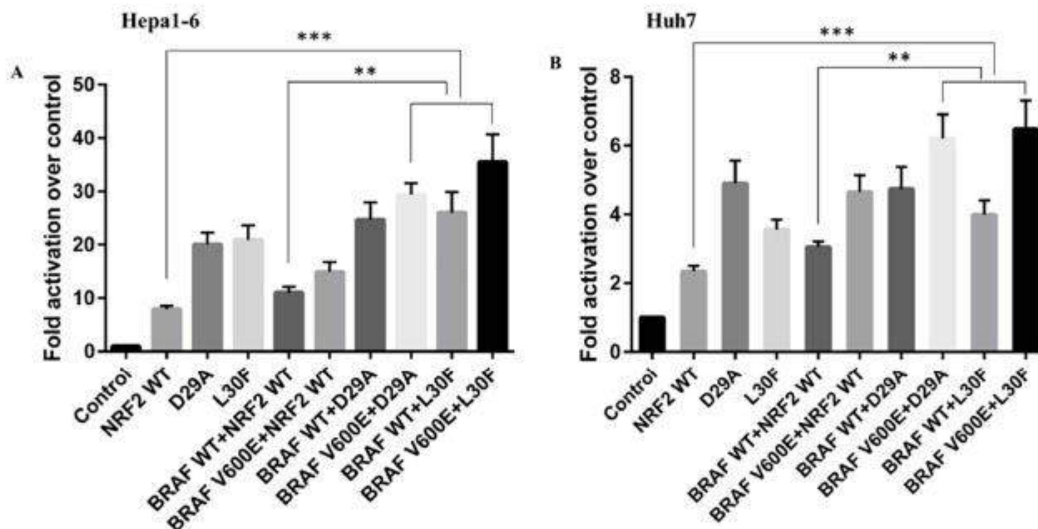


Figure 5. (**A**) The ability of NRF2 WT and MT expression plasmids together with oncogenic BRAF WT and V600E MT expression plasmids to transactivate a NRF2 target promoter (3xARE) in Hepa1-6 cells. (**B**) The ability of the NRF2 WT and MT expreseion plasmids together with oncogenic BRAF WT and V600E MT expression plasmids to transactivate the *MMP9* promoter when overexpressed in Huh7 cells. Data represent the mean ± SEM of 4 independent experiments (**, $p < 0.01$; ***, $p < 0.001$).

Many lines of evidence suggest that BRAF MT is associated with the upregulation of MMP9 expression in several cancers [29,30]. Next, we tested whether BRAF MTs could regulate NRF2 transactivation activity by regulating MMP9 promoter activity in Huh7 cells. We therefore transfected Huh7 cells with a MMP9 promoter reporter in the presence of NRF2 WT or MTs (D29A and L30F) with or without BRAF WT or BRAF V600E. We found that NRF2 MTs overexpressed with BRAF WT showed synergistic induction of MMP9 promoter activity compared to NRF2 MTs alone (Figure 5B). BRAF V600E MT together with NRF2 (D29A, L30F) MTs resulted in an even higher level of NRF2 transcriptional activity as

assessed through the induction of MMP9 promoter activity. MMP9 levels are increased in BRAF V600E expressing tumors [25]. Therefore it was not surprising that MMP9 promoter activity in the presence of NRF2 (D29A and L30F) MTs was found to be lower when BRAF WT was added to the mix when compared to both BRAF MTs together (Figure 5B). Taken together, these data suggested that BRAF MT is synergistically involved with NRF2 MTs in the upregulation of NRF2 transcriptional activity through increased MMP9 transcription.

3. Discussion

Many previous studies have shown that MTs in NRF2 play a role in cancer progression [31–33]. We summarize NRF2 MTs in different cancers and MTs in NRF2 involved with the overactivation of NRF2. The D29A and L30F MTs are found in the DLG motif of the Neh2 domain of NRF2, and this domain is essential for ubiquitination and degradation of NRF2 [34,35]. It has been reported that the structure of the DLG motif is crucial for maintaining proper NRF2 turnover and NRF2-ARE mediated gene expression. Thus, any genetic alteration of *NRF2* might affect its transcriptional activity [36]. Moreover, MTs in the DLG motif change the conserved D29 and L30 residues, which makes it more vulnerable to structural changes [12]. Interestingly, our structural model of NRF2-KEAP1 indicates that the D29A MT in NRF2 drastically reduces its binding affinity with KEAP1, and the L30F MT causes structural hindrance to the pocket in the interface of NRF2 and KEAP1, also resulting in a decrease in binding affinity. Therefore, it is possible that MTs in functional domains of NRF2 may increase the risk of liver cancer by changing their proper structure and function. To this end, our study focused on DLG motif MTs found in several cancers, including HCC [10,37,38]. Previous studies revealed that 6.4% of *NRF2* MTs occur in HCC patients, and constitutive activation of NRF2 occurs more frequently in HCC cases [13,32,37,39–41]. In our study, we observed a constant increase in NRF2 D29A and L30F MT transcriptional activity through antioxidant response element (3xARE)-dependent luciferase reporter gene upregulation. Our findings are in agreement with a previous report of a DEN-induced HCC mouse model that developed DLG MTs in amino acid residues at the position of 29 (80%) and 32 (100%). MTs in those positions were associated with NRF2 overactivation [10]. All the genetic alterations in NRF2 DLG may share a common scenario where all MTs result in the overactivation of NRF2. However, further studies to test the transcriptional activity of all NRF2 DLG MTs are needed to clarify this point.

Under homeostatic conditions, NRF2 is maintained at a very low intracellular concentration through its association with KEAP1 and the Cul3 E3 ligase [42]. Thus, any changes in the DLG motif are vulnerable to KEAP1-dependent polyubiquitination [34]. Moreover, this results in the constitutive activation of NRF2. It has been reported that elevated expression of NRF2 target genes confers advantages in terms of stress resistance and cell proliferation in normal and cancer cells [17]. In our study, we found that KEAP1 co-expression in Hepa1-6 cells visibly reduces the transcriptional activity of NRF2 WT; however, KEAP1 could not reduce the transcriptional activity of NRF2 D29A and L30F MT. Moreover, it has been demonstrated that NRF2 L30F is reductant to KEAP1 mediated protein degradation [12]. These results suggest that NRF2 MTs lead to aberrant transcriptional activity in HCC and induce tumor progression in HCC via upregulation of several NRF2 target genes. In agreement with this, several reports have indicated that both NRF2 DLG and KEAP1 MT can upregulate NRF2 transcriptional activity [12,43].

MMP-9 is important for invasion, metastasis, and tumor angiogenesis [44], and its expression is known to be upregulated in several cancer cells, including HCC [11,44]. The invasion process of MMP9 occurs through its positive correlation with NRF2 and high NRF2 expression in HCC patients associated with a poor prognosis [11]. It has been reported that transcriptional activation of *MMP9* is regulated by NRF2 [18]. It is possible that these phenomena strongly affect the development of malignant phenotypes. Our results suggest that *NRF2* MTs trigger tumor development. The results from our study showed that NRF2 D29A and L30F MTs regulate the transcriptional activity of the *MMP9* promoter through NRF2 induction in Hepa1-6 and Huh7 cells, which suggests that MTs are

linked with the development of HCC. Notably, D29 and L30 are the most frequent *NRF2* gene MTs identified in different human tumors [12,31,32]. However, both D29A and L30F MTs are poorly represented in HCC metastases. We can speculate that overactivation of NRF2 caused by DLG domain MTs in Hepa1-6 and Huh7 cells leads to overexpression of *MMP9* that enhances tumor cell invasion and metastasis. Therefore, aberrant NRF2 expression that increases *MMP9* promoter activity in HCC cells can be considered as a critical target for the development of novel therapeutics.

Oncogenic signaling pathways, including oncogenic *B-RAF* (V619E), have been reported to augment NRF2 transcription via activation of the B-Raf-MEK-ERK and support its pro-tumorigenic effects [21]. Moreover, the activation of BRAF stimulates the transcription of NRF2 via activation of JUN and MYC [21]. In line with this speculation, our study for the first time, provides evidence that the transcriptional activity of NRF2 D29A and L30F MTs were increased with the overexpression of BRAF V600E MT. Recently, one group showed that BRAF MTs altered the tumor microenvironment by regulating the MAPK pathway, and MAPK activation is involved in NRF2 nuclear translocation [45]. Moreover, the overexpression of MAPK pathways is linked with the overexpression of ERK, which leads to the overexpression of several genes involved in tumor development, including *MMP9* [25,45]. Importantly, high levels of MMP9 and BRAF V600E MTs are associated with poor progression-free survival in melanoma patients [25], and activation of NRF2 through this pathway might be critical for tumor cell proliferation. In our study, we have shown a novel molecular mechanism by which BRAF and NRF2 MTs positively regulate transactivation of the *MMP9* promoter in Huh7 cells through NRF2 induction. This mechanism might contribute to HCC cell invasion and metastasis.

As summarized in Figure 6, our results have revealed a critical role played by a NRF2-BRAF-MMP9 signal that could serve as a basis for HCC progression when genes are dysregulated. Our findings could also suggest how MTs in cytoprotective genes can cause aberrant transcriptional activity in a synergistic manner that could lead to the activation of several genes responsible for creating a tumor phenotype. However, the high activity of NRF2 MTs in HCC with BRAF MT warrants further exploration of the potential diagnostic, prognostic, and therapeutic utility of this pathway in HCC.

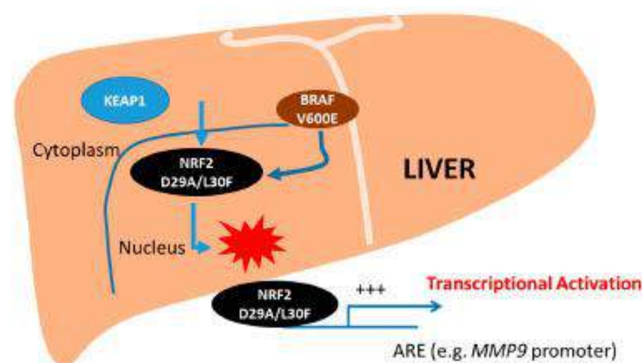


Figure 6. Graphical representation of mutant NRF2 transcriptional activity on target promoters. Novel human NRF2 MTs (D29A, L30F) disturb proper binding to KEAP1 and go to the nucleus, leading to an increase in transcriptional activity. BRAF V600E MT induces NRF2 MT transcriptional activity through increased MMP9 transcription. The increased transcriptional activity caused by NRF2-BRAF-MMP9 signaling may induce cell proliferation and invasion in liver tumors.

4. Materials and Methods

4.1. Cell Culture

Hepa1-6 mouse hepatoma cells (Hepa1-6 cells) and human hepatocyte-derived carcinoma cells (Huh7 cells) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g/L of glucose (Lonza, Basel, Switzerland), 10% fetal bovine serum (FBS) (EURx, Gdansk, Poland), 100 units/mL of penicillin, and 100 units/mL of streptomycin (Lonza, Basel, Switzerland). Cells were maintained under standard conditions: 5% CO_2, temp. 37 °C, humidified atmosphere in the Heracell 150i (Thermo Fisher Scientific, Waltham, MA, USA) incubator. Briefly, the Hepa1-6 and Huh7 cells (2×10^4 cells) were cultured in 24-well plates in DMEM containing 10% FBS and 1% penicillin-streptomycin (Lonza, Basel, Switzerland).

4.2. Plasmids and Primers

Human NRF2 expression plasmid constructs carrying modifications of the WT gene in this study have been published by others and made available through Addgene or from other researchers. These plasmids were human NRF2 WT plasmid (NC16 pCDNA3.1 FLAG NRF2), pcDNA3-HA-KEAP1 (from Dr. Masayuki Yamamoto). Mutant variants of human and mouse NRF2 D29A and L30F were created through site-directed mutagenesis by using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The reporter constructs containing the 3 antioxidant response element (3xARE) promoter in pGL vector were kindly donated by Dr. Raymond J Deshaies. The reporter construct for the *MMP9* promoter was donated by Dr. Thomas Iftner. Reporter assays using these clones were conducted using the DualGlo-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's recommended protocols. Control vectors, pCDNA, and FLAG CMV were also used for control experiments. Specific primers were designed for mutagenesis using the QuikChange Primer Design tool (Agilent Technologies, Santa Clara, CA, USA). Mutated sequences of the DLG motif were confirmed using Sanger sequencing (Genomed, Warsaw, Poland). The primer sequences used for the mutagenesis of the DLG motif were purchased from Genomed (Warsaw, Poland). The primer sequences used for the mutagenesis of the DLG motif were purchased from Genomed (Warsaw, Poland) and are depicted in the following Table 2. Permission for the facility to perform experiments with microorganisms and genetically modified organisms was provided by Minister of the Environment, Poland (Decision number 132/2016).

Table 2. Primer pairs used in our study.

Primer Name	Primer Sequence	Species
D29A	F: CTCGACTTACTCCAAGAGCTATATCTTGCCTC-CAAAGTA R: TACTTTGGAGGCAAGATATAGCTCTTGGAG-TAAGTCGAG	Human
L30F	F: CTCGACTTACTCCAAAATCTATATCTTGCCTC-CAAAGTATGTCA R: TGACATACTTTGGAGGCAAGATATAGATTTTGGAG-TAAGTCGAG	Human
D29A	F: CTCGACTTACTCCAAGAGCTATGTCTTGCCTCCAA R: TTGGAGGCAAGACATAGCTCTTGGAGTAAGTCGAG	Mouse
L30F	F: CGACTTACTCCAAAATCTATGTCTTGCCTCCAAAG-GAT R: ATCCTTTGGAGGCAAGACATAGATTTTGGAG-TAAGTCG	Mouse

4.3. Cell Transfections and Luciferase Assays

To validate the transcriptional activity of NRF2 (WT and MTs), a dual luciferase assay was performed. Hepa1-6 and Huh7 cells (2×10^4 cells/well) were grown to 40–70%

confluency in 24-well plates and transiently co-transfected with the reporter and effector plasmids (that are indicated in Figure legends) with 100 ng of TK-LUC renilla plasmid as an internal control using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Depending on experimental design, we transfected different plasmids accordingly. For KEAP1 co-transfections, 50 ng of KEAP1 plasmid/well was used. Cells were harvested after 48 h and luciferase activity was assayed using a Luciferase Assay Kit (Promega, Madison, WI, USA). Firefly luciferase activity was normalized with Renilla luciferase to control for sample-to-sample variations in transfection efficiency. All reporter assays were repeated independently at least 3 times. Luminescence was measured using a Synergy LX luminometer (Biotek, Winooski, VT, USA).

4.4. Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) of each group in the experiment. The statistical analysis was done using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc tests. Any p-value < 0.05 was considered statistically significant. GraphPad PRISM software version 6 (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ARE	Antioxidant response element
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CMD	Choline- devoid methionine-deficient
CUL3 E3	Cullin 3-RING E3
DEN	Diethylnitrosamine
DMEM	Dulbecco's modified eagle medium
ERK	Extracellular signal-regulated kinases
ESCA	Esophageal carcinoma
FBS	Fetal bovine serum
Hepa 1-6	Mouse hepatoma cell
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG2	Human hepatoma cell line
Huh-7	Human hepatoma cells-7
ICGC	International Cancer Genome Consortium
JUN	Putative transforming gene of avian sarcoma virus 17
KEAP1	Kelch-like ECH-associated protein 1
KO	Knockout

K-Ras	Kirsten rat sarcoma viral oncogene homolog
LUSC	Lung squamous cell carcinoma
MAPK	Mitogen-activated protein kinase
MEK	MAPK or ERK kinases
MMP-9	Matrix metalloproteinase 9
MYC	Cellular homolog of the retroviral v-Myc oncogene
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NSCLC	Non-small cell lung cancer
NRF2	NF-E2-related factor 2
UCEC	Uterine corpus endometrial carcinoma

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HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of *HNF4A* Promoter Activity with Possible Disruption in Transcription Networks

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Abstract: Hepatocyte nuclear factor 1A (HNF1A) is the master regulator of liver homeostasis and organogenesis and regulates many aspects of hepatocyte functions. It acts as a tumor suppressor in the liver, evidenced by the increased proliferation in HNF1A knockout (KO) hepatocytes. Hence, we postulated that any loss-of-function variation in the gene structure or composition (mutation) could trigger dysfunction, including disrupted transcriptional networks in liver cells. From the International Cancer Genome Consortium (ICGC) database of cancer genomes, we identified several *HNF1A* mutations located in the functional Pit-Oct-Unc (POU) domain. In our biochemical analysis, we found that the *HNF1A* POU-domain mutations Y122C, R229Q and V259F suppressed *HNF4A* promoter activity and disrupted the binding of HNF1A to its target *HNF4A* promoter without any effect on the nuclear localization. Our results suggest that the decreased transcriptional activity of HNF1A mutants is due to impaired DNA binding. Through structural simulation analysis, we found that a V259F mutation was likely to affect DNA interaction by inducing large conformational changes in the N-terminal region of HNF1A. The results suggest that POU-domain mutations of HNF1A downregulate *HNF4A* gene expression. Therefore, to mimic the HNF1A mutation phenotype in transcription networks, we performed siRNA-mediated knockdown (KD) of HNF4A. Through RNA-Seq data analysis for the HNF4A KD, we found 748 differentially expressed genes (DEGs), of which 311 genes were downregulated (e.g., *HNF1A*, *ApoB* and *SOAT2*) and 437 genes were upregulated. Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping revealed that the DEGs were involved in several signaling pathways (e.g., lipid and cholesterol metabolic pathways). Protein–protein network analysis suggested that the downregulated genes were related to lipid and cholesterol metabolism pathways, which are implicated in hepatocellular carcinoma (HCC) development. Our study demonstrates that mutations of HNF1A in the POU domain result in the downregulation of HNF1A target genes, including *HNF4A*, and this may trigger HCC development through the disruption of HNF4A–HNF1A transcriptional networks.

Keywords: hepatocellular carcinoma; mutation; HNF1A; POU domain; HNF4A

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1. Introduction

Liver cancer is a major contributor to the cancer burden and one of the leading causes of cancer-dependent deaths worldwide [1,2]. The common risk factors for liver cancer development include alcohol consumption, hepatitis B and C virus infection, and metabolic diseases [3,4]. Most of these factors lead to genetic aberrations in hepatocytes, leading to their oncogenic transformation [3,4]. Researchers studying cancer cell genomes have

undertaken several projects elucidating the genomic alterations present in different cancers, including liver cancer [5–7]. Next-generation sequencing (NGS) analysis data for liver cancer patients in which the most significantly mutated genes were *TP53*, *CTNNB1*, and *TERT* have been reported [7].

Besides these major driver gene mutations, other driver and tumor suppressor genes with well-established roles in liver function have been found. Among many gene mutations found through NGS-based mutational detection, hepatocyte nuclear factor 1A (*HNF1A*) was found to be a frequently mutated gene, one of the top 20 mutated genes in hepatocellular carcinoma (HCC) reported in the International Cancer Genome Consortium (ICGC) database (https://dcc.icgc.org/, accessed on 12 October 2020). HNF1A, a liver-enriched transcription factor, is present in embryonic tissues and plays a pivotal role in cellular differentiation and organ development [8]. *HNF1A* acts synergistically with *HNF4A* to regulate gene expression in various tissues, including the intestine and kidney [9,10]. In addition to its function in liver development, a recent study demonstrated that HNF1A knockout (KO) mice developed HCC due to fatty liver [11]. Moreover, in the HCC microenvironment, HNF1A inhibits Wingless-related integration site (Wnt) and nuclear factor kappa-B (NF-κB) signaling during metastasis and hepatocarcinogenesis [12,13]. On the other hand, the overexpression of HNF1A suppressed the proliferation of HCC and induced the expression of liver-specific genes in HCC cells, which caused cell cycle arrest [14]. These results support the idea that the dysfunction of HNF1A may cause hepatocarcinogenesis and HCC progression. However, while the role of HNF1A in different cancers has recently been examined [15–17], only a few studies have demonstrated a critical link between HNF1A mutations and the development of liver cancer.

Structurally, HNF1A has three domains: a dimerization domain, a DNA-binding domain, and a transactivation domain. The central DNA-binding domain is composed of a Pit-Oct-Unc (POU) homeodomain (POUh) and POU-specific (POUs) domain and is indispensable for efficient transcriptional activity [18]. HNF1A interacts with target DNA as a homodimer or heterodimer with HNF1B to regulate glucose metabolism, lipid metabolism, and detoxification [19–21]. HNF1A occupies the *HNF4A* promoter region and upregulates its expression as positive feedback [22]. Accordingly, a reduction in HNF4A has been associated with the reduced expression of HNF1A in young mice [23]. Similarly, HNF4A and HNF1A, together, form a network that regulates the expression of each as well as multiple liver-specific genes [22,24,25]. Additionally, our group reported for the first time that HNF4A G79C, F83C, and M125I mutations are loss-of-function mutations found in liver cancer patients, leading to a reduction in *HNF1A* gene expression and concomitantly, an increased risk of HCC development [26]. Several studies have demonstrated that HNF1A and HNF4A reciprocally regulate each other's expression through DNA-binding-dependent and independent (protein–protein interaction) mechanisms [27,28]. These findings suggest that both HNF1A and HNF4A are critical regulators of liver function, and their dysfunction leads to liver cancer development. However, unlike for HNF4A mutations, the effects of HNF1A mutations on *HNF4A* gene regulation and HCC development remain elusive. Notably, much like HNF4A mutations, the ICGC and The Cancer Genome Atlas (TCGA) have reported mutations in the DNA-binding domain of HNF1A [29,30]. Previous studies have established that HNF1A mutations are associated with hepatocellular adenomas and maturity-onset diabetes of the young type 3 (MODY3) [30,31]. P112L and Q466X mutations of HNF1A have been associated with MODY [31]. Although mutations of HNF1A Q511L, E32*, and L214Q have also been identified in HCC [30,32,33], the effect of HNF1A POU domain mutation on the regulation of HNF4A and its downstream molecular mechanism to trigger HCC remain unknown.

In our study, we demonstrated that somatic mutations of HNF1A located in the POU domain are possible pathogenic mutations for hepatocarcinogenesis due to their disruption of *HNF4A* gene transcription. The mutations interfere with the ability of HNF1A to bind to the DNA of its target *HNF4A* promoter, and reduced transcriptional activity is observed. Moreover, structural analysis of the HNF1A V259F mutation revealed that it causes large

conformational changes in the N-terminal region. However, RNA-Seq data for HNF4A siRNA knockdown (KD) in human hepatoma cell line (Huh7) cells suggested that the HNF4A mediated decrease in the expression of *HNF1A* and other genes is related to binding activity, the lipid and cholesterol metabolism pathways. These results suggest that proper transcriptional control between HNF1A and HNF4A maintains liver homeostasis and that the disruption of HNF1A–HNF4A transcriptional networks by mutations, aberrant expression or both may play a role in liver cancer development.

2. Materials and Methods

2.1. Cell Culture

Human embryonic kidney cells (HEK293:ATCC CRL-1573) and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/liter of glucose (Lonza, Basel, Switzerland, 10% fetal bovine serum (FBS) (EURx, Gdansk, Poland), 100 units/mL of penicillin, and 100 units/mL of streptomycin (Lonza). The cells were cultured under humidified conditions in an incubator at 5% CO_2 and 37 °C.

2.2. Plasmids and Primers

To amplify the HNF1A sequence, we isolated the genomic DNA from non-immunogenic mouse hepatoma cells (Hepa1–6) using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). The primers for the selected gene were designed based on the sequence located on the chromosome. Restriction sites were incorporated into the forward and reverse primers, respectively. The primers used for cloning the HNF1A plasmid are listed in Supplementary File S1, Table S2. A human HNF1A wild-type (WT) plasmid construct used in this study was procured from Addgene (Teddington, UK). Mutant variants of human HNF1A Y122C and V259F were created through site-directed mutagenesis by using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The *HNF4A* P1 (−985 to +1 of the P1 *HNF4A* promoter) promoter was cloned into a basic pGL3 vector containing the luciferase gene (Promega, Madison, WI, USA) digested with KpnI and HindIII enzymes (Thermo Fisher Scientific, Waltham, MA, USA) using an In-Fusion®HD Cloning Kit (Takara, Shiga, Japan). The reporter constructs P2 (−371 to −37 from the HNF4A transcription start site) and P2-2200 (−2200 to −1 of the P2 *HNF4A* promoter) were purchased from Addgene. CMYC and FLAG CMV vectors were used for control experiments. Specific primers were designed for mutagenesis using the QuikChange Primer Design tool (Agilent Technologies). The Y122C, R229Q and V259F mutated sequences were confirmed using Sanger sequencing (Genomed, Warsaw, Poland). The primers used for the mutagenesis were also purchased from Genomed and are listed in Supplementary File S1, Table S2.

2.3. Reporter Assay

For the reporter assay, 5×10^4 HEK293 cells and Huh7 cells were seeded in 24-well plates. After 24 h, the cells were transfected with 100 ng of the mouse and human plasmids indicated in the figures, using Lipofectamine 3000 (Thermo Fisher Scientific). The cells were transiently co-transfected with 500 ng of an *HNF4A* promoter–reporter construct containing consensus binding sites upstream of the firefly luciferase and 100 ng of a thymidine kinase promoter-Renilla luciferase reporter plasmid, as an internal control, using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. Following 48 h of transfection, the cells were lysed and the luciferase activity was measured with a Luciferase Assay Kit (Promega), according to the enclosed protocol, using a Synergy LX luminometer (Biotek, Winooski, VT, USA).

2.4. Western Blotting

A total of 5×10^5 HEK293 cells were plated in 6-well plates and transfected for overexpression with different HNF1A plasmids in amounts of 2 µg for 48 h, using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. The nuclear protein concentrations from the HNF1A WT and HNF1A mutant cells were determined

using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The molecular weight of the protein was estimated with Precision Plus Protein WesternC Standards (Bio-Rad, Hercules, CA, USA). A total of 10 μg of each protein sample was loaded on an SDS-polyacrylamide gel (4% stacking gel; 12% resolving gel), separated, and transferred to a PVDF membrane (Merck Millipore, Burlington, MA, USA) by wet transfer. The membranes were blocked with 5% skim milk and then incubated with the antibodies. The blot was incubated overnight with mouse monoclonal Anti-Flag antibody (1:5000, Sigma) in 1% skim milk and 0.1% PBST at 4 °C, followed by incubation with HRP-conjugated anti-mouse IgG produced in goats (1:5000, Sigma-Aldrich, Saint Louis, MO, USA) in 1% skim milk and 0.1% PBST for 1 h at room temperature. For the siRNA KD experiment, we used rabbit monoclonal anti-HNF4A (1:1000, Cell Signaling Technology, Danvers, MA, USA) antibody and anti-rabbit IgG produced in goats (1:5000, Sigma-Aldrich). Anti-β-actin (1:1000, Cell Signaling Technology) was used as a loading control. The proteins were visualized using an ECL Western Blotting Analysis System (Amersham, Illinois, CA, USA) and ChemiDoc XRS + System (Bio-Rad, Hercules, CA, USA).

2.5. *Immunofluorescence (IFC)*

For IFC staining, 5×10^5 HEK293 cells were plated in 6-well plates and transfected for overexpression with HNF1A WT and mutant plasmids in amounts of 2 μg for 48 h using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. After that, the cells were fixed by incubating them in 4% paraformaldehyde for 15 min at room temperature. After washing the cells with PBS 0.1% Tween-20 (PBST), the cells were treated with PBS 0.5% Tween-20 (PBST) for 10 min. Next, the cells were blocked in 1% skim milk for 20 min at room temperature. The cells were washed with PBS 0.1% Tween-20 (PBST) and incubated overnight at 4 °C with mouse monoclonal FLAG-antibody. Then, the cells were washed with PBST and incubated with Alexa546-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. After the cells had been washed 3 times with PBST, the cell nuclei were counterstained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min. The cells were finally washed with PBS and mounted on slides with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). The cells were observed under a confocal microscope (A1R, Nikon, Tokyo, Japan) equipped with 10x, 20x, 40x and 60x lenses; Nomars- 5 ki's DIC contrast; Hoffman's modulation contrast; 405-, 488-, 561- and 640-nm lasers; a hybrid scanner; and a resonance scanner (Nikon). The workstation was equipped with Nikon's Confocal NIS-Elements package. The confocal images were analyzed using the IMARIS 6.0.1 software (Bitplane AG, Oxford, UK).

2.6. *Electrophoretic Mobility-Shift Assay (EMSA)*

Oligonucleotides synthesized by Sigma-Aldrich were used for DNA-binding assays. Sequence information is provided in Supplementary File S1, Table S2. Generation of double-stranded probes were done by heating equal molar amounts of each of the 5′ to 3′ oligonucleotides with their respective complementary oligonucleotides at 95 °C for 10 min, followed by cooling at room temperature. Next, double-stranded oligonucleotides were labeled with DIG-11-ddUTP using recombinant terminal transferase (20 units/mL) in a final volume of 25 μL, according to the DIG Gel Shift Kit, second generation manufacturer's instructions (Roche Applied Science, Mannheim, Germany). EMSA was performed according to the manufacturer's directions. In brief, DNA-binding reactions were set up using 10 μg of a nuclear extract of either WT or mutant proteins. These proteins were mixed with the above-mentioned DIG-labeled oligonucleotides in a DNA-binding buffer containing 1 μg of poly(dI-dC) and 0.1 μg of poly-l-lysine, in a final reaction volume of 20 μL.

2.7. *Molecular Dynamics (MD) Simulations*

The structure of the POUh domain (residues 201 to 278, chain B) of HNF1A was derived from the Protein Data Bank; the ID is 1IC8 [34]. Protein and water molecules

within 5 Å of the POUh domain were retained and considered in the initial structure. The N-terminal residue of the protein was capped with an acetyl group to reduce the truncated effect of the POUs domain. Hydrogen atoms were added to the protein and water molecules with the pdb2gmx module of GROMACS [35] under the assumption of the standard protonated state. The simulation system was solvated with TIP3P water molecules [36] and neutralized in a dodecahedron box with a minimum distance of 12.0 Å between the protein and the box edges and with 0.15 M concentrations of Na^+ and Cl^- ions. The AMBER ff14SB parameter set [37] and the parameter set previously reported [38] were employed for the force fields of the protein and Na^+ and Cl^- ions, respectively. The total number of atoms in the box was 29,144. The V259F mutant's structure was modeled using MODDELER [39]. In the process, residues within 8 Å of the C_β atom of V259 could move to avoid any atomic overlap. The mutant system was also prepared as the WT system above. The total number of atoms in the box was 29,154.

All the MD simulations were performed with GROMACS. Ten independent runs were performed as follows: the simulation systems were first subjected to energy minimization with the steepest descent method, followed by the conjugate gradient method. Then, for equilibrating the systems, MD simulations were carried out for 100 ps at 300 K with NVT condition and for another 10 ns at 300 K with NPT condition using Berendsen's method [40]. Finally, for each system, product runs were carried out for 200 ns at 300 K under NPT condition using the Parrinello–Rahman method [41]. The temperature was maintained with Langevin bath (the time constant for coupling was 2 ps) [42], and the electrostatic interactions were calculated with the particle mesh Ewald method [43]. Non-bonded interactions were cut off at 10 Å, and the bond length including hydrogen atoms was constrained by LINCS method [44] for protein, and the SETTLE method [45] for the water molecules. The integral time step was set to be 2 fs. For analysis, the last 100 ns trajectories were used. The total MD trajectory for analysis was 1 μs.

Residue-wise intra-contact was counted if any of the heavy atoms from a pair of residues was less than 4.5 Å. Then, the differences in contacts between the WT and V259F mutant were calculated by subtracting the contacts of V259F from those of the WT. The last 100 ns of all the 10 trajectories were used for the contact-map calculation. The solvent-accessible surface area was calculated with VMD [46]. The molecular figures were also created with VMD [46].

2.8. *KD by HNF4A siRNA*

For KD, a total 3×10^5 Huh7 cells were plated in 6-well plates and transfected with a 20 nM concentration of either control or HNF4A siRNAs using the Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher) according to the manufacturer's instructions and cultured for 48 h in DMEM + 10%FBS medium without antibiotics. The sequences of the siRNAs and primers are listed in Supplementary File S1, Table S2, and MISSION siRNA Universal Negative Control (SIC-001-s) was obtained from Sigma Genosys (Sigma Genosys Holdings LLC, TX, USA). After 48 h of transfection, the cells were lysed with T-PER for the extraction of whole cell protein, and Western blotting was performed as described above.

2.9. *RNA-Sequencing (RNA-Seq) and Functional Analysis*

Total RNA was extracted from siRNA KD Huh7 cells with the NucleoSpin®RNA kit (MACHEREY-NAGEL, Düren, Germany). For the reverse transcription, 0.5 μg of total RNA was used and the reactions were performed according to the manufacturer's protocol (EURx, Gdansk, Poland). PCR was performed with the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Waltham, MA, USA) using the GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA). GAPDH expression was utilized for normalization. RNA-Seq was then carried out via a commercially available service (service ID# F21FTSEUHT1601, BGI, Huada Gene, Wuhan, China). We analyzed the RNA-Seq data for two HNF4A siRNA KD (2 replicates) Huh7 cell samples. The KEGG enrichment path-

way and GO bioinformatic analyses were conducted using BGI's Dr. TOM approach, an in-house customized data-mining system of the BGI. The average of 2 controls and average for the KD (2 siRNA1 and 2 siRNA2) were used to calculate the differential gene expression. The upregulated or downregulated expression of genes was expressed as log2FC, which represents the log-transformed fold change (log2FC = log2[B] − log2[A]).

2.10. Statistical Analyses

The data are presented as the means ± standard errors of the means (SEMs) for each group in the experiment. The statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. P values less than 0.05 were considered to indicate statistical significance. The GraphPad PRISM software version 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis.

3. Results

3.1. Somatic Mutations Found in the Functional Domain of HNF1A

Next-generation sequencing has helped to decipher the low-frequency somatic mutations of HCC and identified HNF1A as a candidate driver gene [29]. HNF1A mutations mostly located in the POU domain of HNF1A, identified in the ICGC database, are presented here (https://dcc.icgc.org/, accessed on 12 October 2020). The POU-domain mutations reported in different liver cancer projects are listed in Figure 1A and Table 1. The data suggest that HNF1A mutations in these regions may have an impact on hepatocarcinogenesis. In our study, we performed functional analyses of three mutations (Y122C, R229Q and V259F) located in the POUs and POUh domains (Figure 1A). From an evolutionary perspective, the mutant amino acid residues are strictly conserved among various species (Figure 1B); the asterisks in red specify the locations of the POU domain mutations (Y122C, R229Q and V259F). The conserved domains among the different species (humans, mice, bovines and zebrafish) are highlighted in red, and the domains that we functionally analyzed are 100% conserved throughout the different species. The mutations in such evolutionarily conserved elements might have a strong effect on the protein function and warrant further investigation.

Table 1. HNF1A mutations located in POU domain found in liver cancer patients.

Mutation ID	Genomic DNA Change	Type	Consequences	Project in Which Mutation Observed	Conservation among Species
MU854410	chr12:g.121432028G>T	Single base substitution	V259F	LINC-JP	YES
MU837628	chr12:g.121426674A>G	Single base substitution	Y122C	LINC-JP	YES
MU81565444	chr12:g.121431445G>T	Single base substitution	A217S	LICA-CN	YES
MU85877851	chr12:g.121426663G>T	Single base substitution	M118I	LICA-CN	YES
MU20638	chr12:g.121431482G>A	Single base substitution	R229Q	LICA-FR	YES
MU29769426	chr12:g.121431410A>C	Single base substitution	K205T	LICA-FR	YES
MU82396333	chr12:g.121426664G>T	Single base substitution	V119F	LICA-CN	YES
MU602436	chr12:g.121426701G>T	Single base substitution	R131L	LICA-CN	YES
MU29793014	chr12:g.121431983A>G	Single base substitution	R244G	LIHC-US	YES

Table 1. *Cont.*

Mutation ID	Genomic DNA Change	Type	Consequences	Project in Which Mutation Observed	Conservation among Species
MU29769474	chr12:g.121431977A>T	Single base substitution	I242F	LICA-FR	Not in Zebrafish
MU128970370	chr12:g.121426782A>G	Single base substitution	K158R	LIHC-US	YES
MU85320917	chr12:g.121431501G>T	Single base substitution	E235D	LICA-CN	YES
MU128971993	chr12:g.121431424T>A	Single base substitution	S210T	LIHC-US	YES
MU29496420	chr12:g.121432040C>G	Single base substitution	R263G	LIHC-US	YES
MU29433874	chr12:g.121432014T>A	Single base substitution	L254Q	LIHC-US	YES
MU29746856	chr12:g.121431506A>G	Single base substitution	N237S	LIHC-US	YES
MU822656	chr12:g.121431466C>G	Single base substitution	P224A	LIAD-FR	YES
MU822434	chr12:g.121431413G>T	Single base substitution	W206L	LIAD-FR	YES
MU822864	chr12:g.121432067C>A	Single base substitution	R272S	LIAD-FR	YES
MU823044	chr12:g.121432041G>T	Single base substitution	R263L	LIAD-FR	YES

3.2. *HNF1A Mutants Display Reduced Transcriptional Activity and Decreased Binding Ability*

It has been reported that HNF1A mutations affect DNA binding and reduce the transcriptional activity. However, there are few reports on the functional analysis of disease-associated mutations in HNF1A [30,32]. Thus, we sought to determine how the novel mutations found in the POU domain affected the properties of the mutant proteins and impaired the transcriptional ability of HNF1A. To evaluate the effects of these somatic mouse (Y122C, R229Q and V259F) and human HNF1A (Y122C and V259F) mutations in the POU domain, we examined the transcriptional activity of those mutants found in liver cancer patients. We compared the ability of the human and mouse HNF1A mutant proteins to transactivate HNF1A-responsive elements containing the *HNF4A* P-1 promoter (Figure 2A,B).

The overexpression of human and mouse WT HNF1A stimulated the transcription of HNF1A-responsive element-containing promoters; however, Y122C, R229Q human and Y122C mouse mutations resulted in a decreased transactivation function for HNF1A toward *HNF4A* P1 (Figure 2A,B). More importantly, the mouse and human HNF1A V259F mutations completely lost their transcriptional activity in all cases (Figure 2A,B). In our study, we found a similar effect of the HNF1A mutants on the *HNF4A* P2 promoter (Supplementary File S1, Figure S1A–D). With Huh7 cells, which endogenously express HNF1A, we found that HNF1A WT had higher transcriptional activity, but both mutations (Y122C and V259F) resulted in reduced transcriptional activity for the *HNF4A* P1 promoter (Figure 2C), and similar activity was also found in the case of the *HNF4A*-P2 promoter (Supplementary File S1, Figure S1E). These results are consistent with a previous study indicating that MODY3-associated mutants displayed reduced transcriptional activity for their target promoter [47,48]. Therefore, our functional analysis revealed that the mutations in the POU domain cause reduced HNF1A transcriptional activity, suggesting that the mutations located in this domain merit further study.

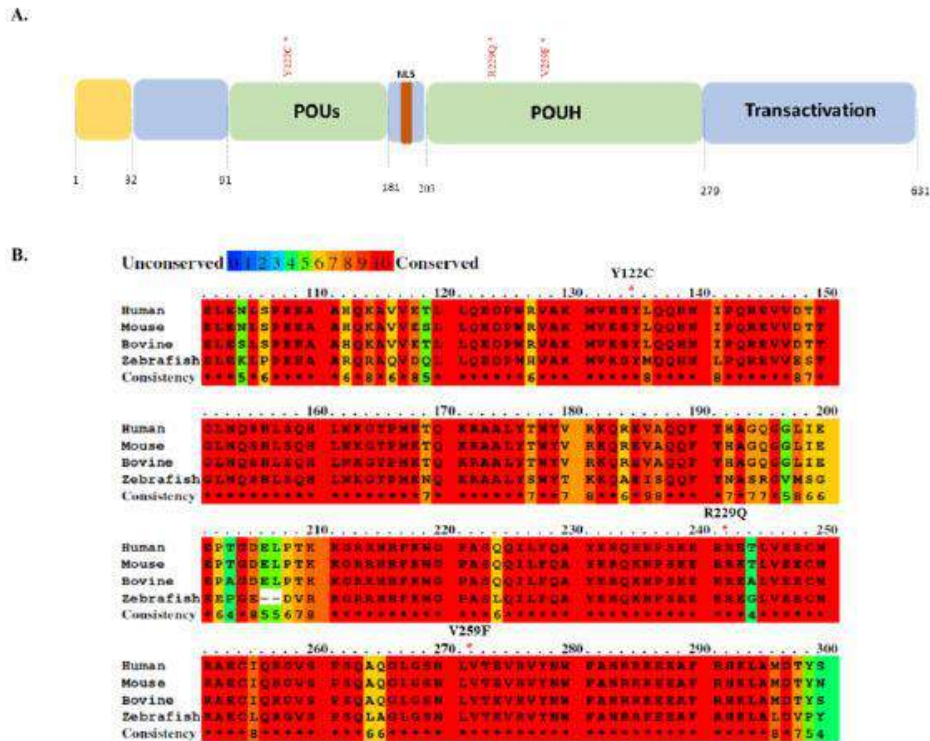


Figure 1. (**A**) Positions of novel mutations are indicated in the human HNF1A protein structure (Pit1, Oct1 and Unc1 (POU) domain-Green; POU homeodomain (POUh) and POU-specific (POUs)). (**B**) Alignment of the human, mouse, bovine and zebrafish HNF1A amino acid sequences and mutations found in the POU domain. Red color denotes highly conserved (100%) elements among the species. The mutations (indicated by red asterisks) in the POU domain of HNF1A are highly conserved in species.

As most of the somatic mutations analyzed in our study are localized in the POU domain (Figure 1A), we investigated the DNA-binding ability of the mutant HNF1A proteins. Furthermore, reduced transcriptional activity suggests that mutations may directly affect the DNA-binding ability of HNF1A. Using the EMSA, we measured the DNA-binding affinity of WT and mutant HNF1A proteins. We found a clear correlation between the effects of these mutations on HNF1A transcriptional activation and DNA binding. HNF1A Y122C, R229Q and V259F mutants exhibited markedly reduced binding to the *HNF4A* promoter compared to the WT HNF1A (Figure 2E), whereas the WT and mutant HNF1A proteins were expressed equally, as demonstrated by Western blot (WB) analysis (Figure 2F). Changes in the nuclear localization of proteins may affect transcriptional activity. Therefore, we analyzed whether mutations of HNF1A (Y122C, R229Q and V259F) affected its proper nuclear localization ability. IFC staining revealed that both the WT and mutant HNF1A were localized in the nuclei of HEK293 cells (Figure 2D). Thus, our findings strongly suggest that HNF1A Y122C, R229Q and V259F mutants have reduced transcriptional activity due to the loss of their ability to bind to *HNF4A* promoter regions, and these are related to the loss of HNF4A expression and function. Notably, the RNA-Seq data obtained from The Cancer Genome Atlas (TCGA) database of cancer patients showed that the expression

of *HNF4A* and *HNF1A* mRNA is significantly correlated in many cancer types (Supplementary File S1, Figure S3). These results suggest that HNF1A and HNF4A are involved in a cross-regulatory network, and if a loss-of-function mutation occurs in one, it may lead to the reduced expression of the other. In our previous study, we found that HNF4A Zn-finger mutations resulted in a similar phenotype and that the *HNF1A* promoter could not bind with the HNF4A G79C mutant, partially due to the disrupted fluctuation of the protein structure ([26], Supplementary File S1, Figure S2A–C). Therefore, we further investigated whether this type of structural change occurred when the HNF1A POU domain was mutated.

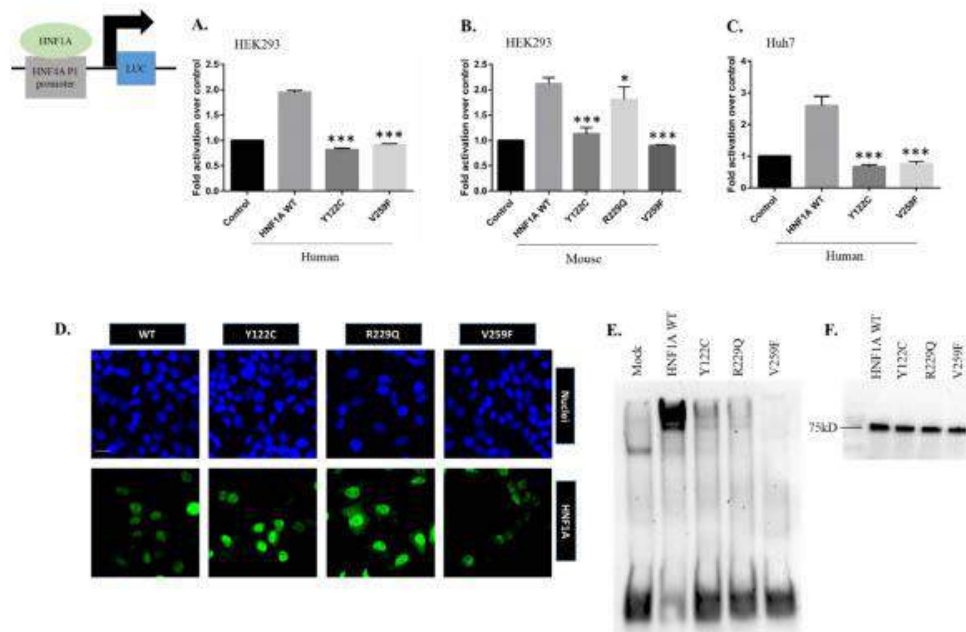


Figure 2. The ability of the (**A**) human WT and mutant HNF1A and (**B**) mouse WT and mutant HNF1A to transactivate *HNF4A* P1 when overexpressed in HEK293 cells. (**C**) The ability of the human WT and mutant HNF1A to transactivate the target promoter (*HNF4A* P1) when overexpressed in Huh7 cells. The cells were co-transfected with the indicated luciferase reporters and either an empty expression vector (serving as a control) or expression vectors (100 ng) for the indicated HNF1A vectors in 24-well culture plates. The bars indicate the fold activation of HNF1A WT and mutants (vs. control) on target promoters. The corresponding promoter activity is reported as fold activation over control (±SEM, $n = 3$–4). The data reported represent the averages of three experiments, each conducted in duplicate. (*, $p < 0.05$; ***, $p < 0.001$). (**D**) Cellular localization of WT and mutant HNF1A was visualized in HEK293 cells using IFC staining. The nuclei were stained with DAPI, and the images were taken at 20× magnification. (**E**) Electrophoretic mobility shift assay (EMSA) was used to assess the binding of WT or mutated HNF1A nuclear proteins to a double-stranded oligonucleotide corresponding to the consensus HNF1A-binding elements of the *HNF4A* promoter region. The HNF1A V259F mutant displayed markedly reduced binding to the *HNF4A* promoter region for all the experiments. (**F**) HEK293 cells were transfected with expression vectors encoding HNF1A WT or the indicated mutants. WB analysis showed that all proteins were similarly expressed.

3.3. Dynamics of the HNF1A V259F Mutant Revealed That the Mutation Affects Protein Stability and Causes Rearrangement in the N-Terminal Region

V259 is located in the POUh domain, related to the DNA-binding region of HNF1A, and is thus considered functionally important (Figure 3A). It should be noted that V259 is not directly involved in protein–DNA interactions, but the mutation has been found to reduce the binding affinity. To examine the impact of the mutation, we conducted 10 independent all-atom MD simulations with explicit solvent models for each of the WT and V259F proteins. The root-mean-square fluctuations (RMSFs) showed that the fluctuations of the structures were similar, except for the N-terminal region, in which they differed (Figure 3B). V259F had a significantly larger fluctuation than the WT at the N-terminal but not in other regions, including the DNA-recognition helix (residues 260 to 274) and mutation site.

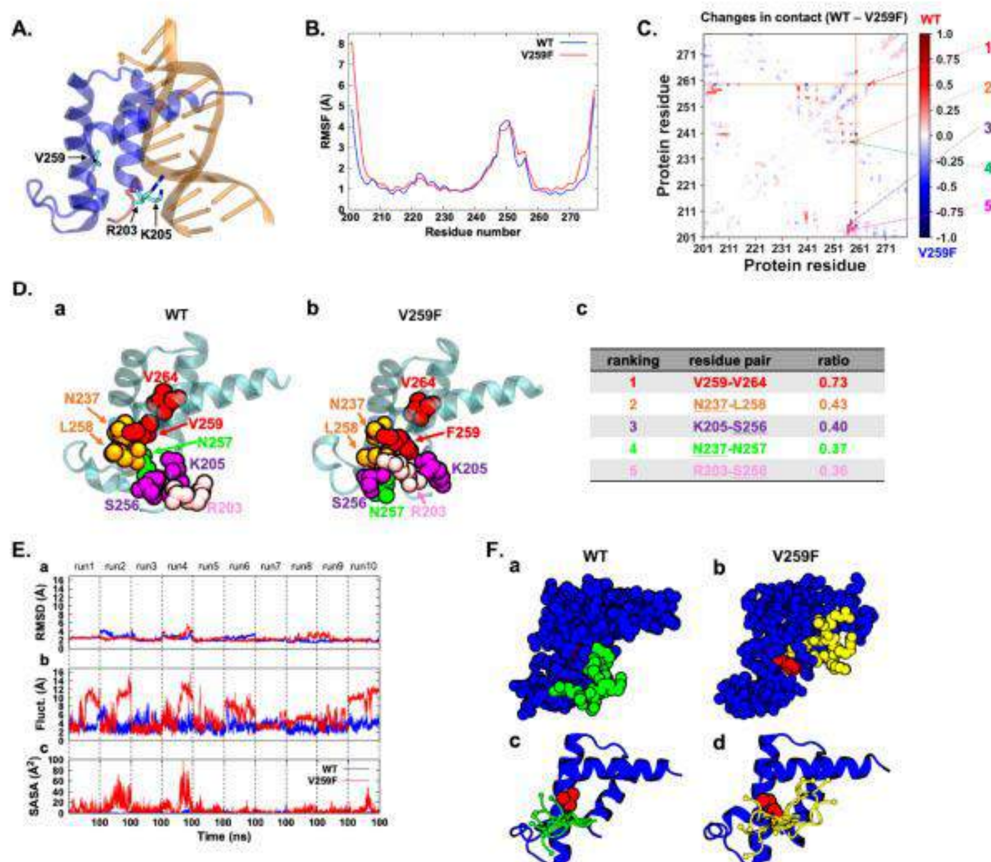


Figure 3. **(A)** X-ray crystallographic structure of the POUh domain (blue) binding with DNA (orange; PDB ID: 1IC8). The backbone of the N-terminal region is highlighted in red. The residues V259, R203

and R205 are depicted by the stick model. (B) Fluctuations of the WT (blue) and V259F mutant (red) proteins. The RMSF of the backbone was plotted. The fluctuations were calculated using the last 100 ns of 10 runs (1 μs in total). (C) Residue-wise contact-map difference between the WT and V259F mutant proteins. The contacts were calculated using the last 100 ns of 10 runs (1 μs in total). The colors denote the ratios of contact in the simulation time. The five most contacted residue pairs in WT proteins are denoted with numbers. We defined contact as any heavy atom of the residue-pair within 4 Å. (D) (a) The WT structure closest to the averaged structures over 1 μs trajectories. The protein backbone is drawn in a ribbon representation (cyan). The heavy atoms of the five most contacted residue pairs are depicted with a space-filling model; see also (c). (b) The V259Fmutant structure closest to the averaged structures over 1 μs trajectories. The heavy atoms of the five most contacted residue pairs in the WT are depicted in the space-filling model. The residue pairs in contact in the WT were completely lost. (c) Contact ratio of residue pairs in the 1 μs trajectories. The residues in the list are shown in (a,b) with distinct colors. Underlined residues appeared twice in the list. (E) (a) Backbone-RMSD of the whole POUh domain except for the N-terminal region (residues 201 to 206) against the X-ray crystallographic structure. (b) Fluctuation of the N-terminal region. The fluctuation was calculated using the RMSD-fitted structures of (a). (c) Solvent-accessible surface area (SASA) of the sidechain atoms of residue at position 259: Val of the WT is depicted in blue, and mutated residue Phe, in red. Dotted lines show the boundaries of runs. (F) A typical snapshot of the WT (a) and V259F mutant proteins (b) in the trajectory. The 259th residue and the N-terminal region are depicted by red and green/yellow, respectively. (c) The conformations in the N-terminal region of the WT. The images depict 10 structures taken from the last snapshots of 10 runs in green, and V259 is denoted by red in the space-filling model. (d) The conformations in the N-terminal region of the V259F mutant. The images depict 10 structures taken from the last snapshots of 10 runs in yellow, and F259 is denoted by red in the space-filling model.

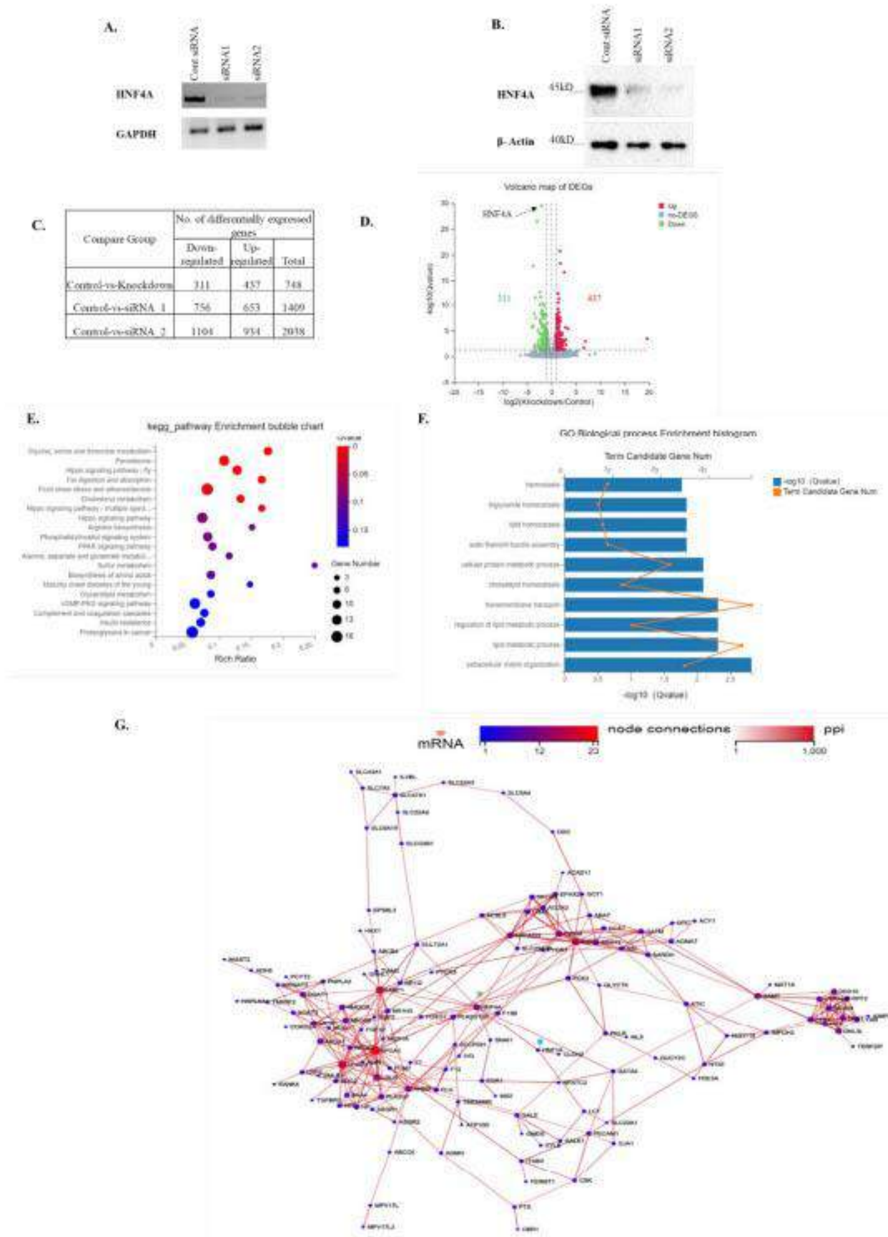
We further investigated why this large fluctuation occurred in the N-terminal region. The residue-wise contact map illustrates the changes in the interaction between the two residues. The map shows that V259F lost several key interactions: the hydrophobic interaction of V259–V264 and electrostatic and/or hydrophobic interactions of N237–L258, K205–S256, N237–N257 and R203–S256 (Figure 3C,D). The loss of these interactions destabilized the hydrophobic packing formed around V259 in the WT.

As seen in Figure 3E,F, in the WT structure, the 259th residue Val was nearly always shielded from the solvent. In the mutant structure, the mutated Phe was often exposed to the solvent. We observed a correlation between the solvent-accessible surface area of the Phe and the fluctuation in the N-terminal region (Figure 3E,F). This suggests that the N-terminal region managed to shield the Phe from the solvent, but that conformation was unstable, thereby causing the large fluctuation in the N-terminal region. These large conformational changes in the N-terminal region result in the loss of DNA interactions by R203 and K205, reducing the DNA-binding affinity. Furthermore, this fluctuation affects the arrangement of the POUh and POUs domains, both of which bind to DNA.

3.4. siRNA KD of HNF4A Causes Differential Gene Expression and Overrepresented Pathways

Overall, the results suggest that POU domain mutations of HNF1A downregulate *HNF4A* gene expression. Therefore, to mimic the HNF1A mutation phenotype in transcription networks, we performed siRNA-mediated KD of HNF4A. Two pairs of oligonucleotides encoding HNF4A-specific siRNAs were designed to silence HNF4A expression. After 48 h of transfection, the HNF4A levels were significantly decreased in Huh7 cells through HNF4A siRNA treatment (Figure 4A). We also examined the changes in HNF4A protein levels in Huh7 cells, which endogenously express high levels of the HNF4A protein. The HNF4A siRNA markedly reduced the HNF4A protein levels as compared with the controls (Huh7 cells transfected with the control siRNA; Figure 4B). HNF4A is a known tumor suppressor, regulating the transcription of a myriad of genes [10,25,26]. To further understand the effect of KD on the mechanism underlying HNF4A's tumorigenic function, RNA-Seq analysis was performed to evaluate the genome-wide gene expression profile in

HCC cells after HNF4A KD. RNA-Seq data analysis revealed that 748 genes were differentially expressed in the HNF4A KD cells (Figure 4C). We found a distinct difference in the global gene expression profile in control versus KD cells; among 748 genes, 311 genes were downregulated and 437 were upregulated (Figure 4D). The KD of HNF4A resulted in the down- and upregulation of many genes known to be involved in transcriptional regulation (Supplementary File S2). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that the most overrepresented pathways were the Hippo signaling pathway, and the lipid and cholesterol metabolic pathways (Figure 4E). Gene ontology (GO) analyses revealed that the genes were largely involved in biological processes, such as lipid and cholesterol metabolism, and extracellular matrix organization (Figure 4F). GO analysis also showed that the genes were involved in molecular functions, such as binding activity (e.g., protein, cholesterol, actin filament, and signaling receptor binding; Supplementary File S1, Figure S4). The protein–protein interaction analysis of the downregulated genes revealed that HNF4A downregulation also caused the downregulation of its target proteins, which are involved in lipid and cholesterol metabolism (Figure 4G).



Compare Group	No. of differentially expressed genes		
	Down-regulated	Up-regulated	Total
Control-vs-Knockdown	311	437	748
Control-vs-siRNA_1	756	653	1409
Control-vs-siRNA_2	1104	934	2038

Figure 4. HuH7 cells were transfected with 20 nM concentrations of either control siRNA (SIC; MISSION®siRNA Universal Negative Control #1) or HNF4A-specific siRNAs. (**A**) The KD efficiency

was verified by RT-PCR. (B) Western blot analysis shows that HNF4A expression was decreased in HNF4A-siRNA-treated HuH7 cells. (C) The number of differentially expressed genes (DEGs) in HNF4A-knockdown (KD) cells. (D) Volcano plot map representing DEGs in KD cells. Red dots represent upregulated genes, green dots show downregulated genes, and gray represents non-DEGs in KD cells. (E) Top 20 KEGG pathways in KD cells. (F) GO analyses of the top 10 biological processes. (G) Protein–protein interaction of downregulated genes. Green asterisk indicates HNF4A, and blue asterisk indicates HNF1A in the network.

4. Discussion

Recent advances in NGS technologies have identified major cancer-driving genes in the liver, and their mutations are related to liver carcinogenesis [5,49]. Previously, HNF1A mutations were identified in diabetes, and their functional effect was validated [31,50,51]; however, only very few studies have suggested that HNF1A mutations identified in HCC are associated with the development and progression of HCC [32]. Interestingly, we found that HNF1A was one of the genes commonly found to be mutated in HCC according to the ICGC database (https://dcc.icgc.org/, accessed on 12 October 2020), and many mutations are accumulated in the POU domain of HNF1A (Table 1). On the other hand, HNF4A is also known as a major tumor suppressor, and its expression is tightly regulated by HNF1A [26–28]. These findings suggested that the role of HNF1A POU domain mutations in the regulation of HNF4A expression in the context of HCC molecular pathology merited investigation. In this study, we demonstrated the functional effect of POU domain mutations of HNF1A on HNF4A gene regulation and investigated their effects on alterations in transcriptional networks through the dysregulation of HNF4A gene expression.

The HNF family harbors common features such as DNA-binding and transactivation capabilities that account for its functional diversity [29,52]. HNF family gene mutations are mostly known to occur in the functional domain of the protein and inhibit the protein's activity by affecting its DNA-binding affinity and protein conformation [26,53]. Our study presents a systematic analysis of the ICGC database of HNF1A transcription factor (DNA-binding protein) mutations in the POU domain (Table 1). Notably, the HNF1A mutations reported in this domain are highly conserved among different species (Figure 1B). We studied three substitution mutations (Y122C, R229Q and V259F) in the POUs and POUh domains (Figure 1A). The results imply that the HNF1A mutations we identified in the POU domain are pathogenic mutations that strongly affect protein function and augment the risk of the initiation of liver cancer development. Previous studies have demonstrated that the R271W and S247T mutations of HNF1A located in the POUh domain impair HNF1A's transcriptional activity to transactivate the *HNF4A* promoter [48,54]. These results are consistent with the data from our study, in which we found impaired transcriptional activity of HNF1A Y122C, R229Q and V259F mutants in the regulation of *HNF4A* promoter activity. Moreover, the HNF1A Q511L mutation was reported to reduce the function of HNF1A to regulate *HNF4A* promoter activity as well as to inhibit the proliferation, migration, and invasion of HCC cells [32]. Therefore, our results suggest that reduced *HNF4A* promoter activity caused by HNF1A POU-domain mutations may play a role in HCC development. Loss-of-function mutations caused by substitution or deletion represent the majority of functionally characterized MODY mutations [31,34]. In fact, several functionally validated HNF1A mutations have been found in MODY patients [47,48,55]. Apart from the mutations verified in this study, we found several HNF1A mutations located in the POU domain (Table 1). While our study emphasizes the importance of POU-domain mutations of HNF1A, further functional studies are needed to verify the mutations found in different countries. Similarly, since HNF1A mutations are commonly found in MODY patients, it is clinically important to verify the risk of liver cancer development in MODY patients.

It is known that in mice the hepatocyte-specific deletion of HNF1A leads to the spontaneous development of HCC due to fatty liver without cirrhosis [11]. Moreover, the hepatocyte-specific deletion of HNF1A in mice leads to non-alcoholic steatohepatitis (NASH) and HCC [11]. Similarly, the KO/KD of the major HNF1A target gene *HNF4A* is

known to play a role in liver oncogenesis or HCC [56–58], suggesting that both HNF1A and HNF4A are responsible for maintaining liver homeostasis, and the disruption of their function may lead to liver pathologies and HCC. In our study, we observed that HNF1A Y122C, R229Q and V259F mutations significantly decreased the transcriptional activity regarding the regulation of the *HNF4A* gene and reduced the DNA-binding capacity of HNF1A for the *HNF4A* promoter. Conversely, the HNF4A G79C mutation reduced the ability of HNF4A to bind to the *HNF1A* promoter (Supplementary File S1, Figure S2B). We and others have suggested that HNF1A and HNF4A are involved in a regulatory network [26,59,60] and that their gene expression is tightly correlated [60,61] (Supplementary File S1, Figure S3); as such, pathogenic mutations in either the *HNF1A* or *HNF4A* gene may increase the risk of HCC by reducing their expression. In fact, a few studies have revealed that the HNF1A–HNF4A axis is an important pathway for the control of liver homeostasis and that its disruption can cause liver cancer. However, further in vivo studies are needed to clarify the importance of these possible pathogenic mutations in HCC.

The loss-of-function V259F mutation in HNF1A was subjected to rigorous structural and stability analyses to identify its deleterious effect. MD simulations allowed us to elucidate the dynamic nature of the protein–DNA interaction when the mutation occurred at an atomic level (Figure 3D). As demonstrated by Sneha et al. [53], a higher RMSF is associated with reduced stability, consistent with our observation that the V259F mutant complex exhibited a greater fluctuation pattern (Figure 3C), which was correlated with a reduction in the number of intermolecular hydrogen bonds formed in the V259F mutant complex compared with the WT HNF1A complex (Figure 3A,C). It is known that proteins have arginine residues on their surfaces, which greatly increases the proteins' stability [62]. By contrast, the rearrangement of arginine and lysine residues results in reduced stability and negatively affects the protein function. However, according to our WB experiment, the HNF1A protein and mutants are equally expressed. The complete loss of DNA binding for V259F suggests that valine is an essential base that is important for DNA interaction and DNA-binding affinity. It has been reported that the disruption of helix 3 (residues 260 to 274) during substitution mutations could cause a conformational change in the protein and affect the protein's function [47]. In line with this, it is postulated that V259F changes the conformation of the HNF1A protein's structure and gives rise to an unstable structure in the N-terminal region. Altogether, we conclude that the large conformational changes in the N-terminal region, but not the change in protein stability, resulted in the loss of DNA interactions by R203 and K205, reducing the DNA-binding affinity.

In this study, we found that loss-of-function mutations of the HNF1A POU domain trigger a reduction in HNF4A gene expression. However, the molecular mechanism through which the loss of function may cause disrupted gene expression and, therefore, promote HCC at the molecular level remains to be understood. To determine the molecular mechanisms, we performed a global gene expression analysis in the condition of HNF4A KD. The top seven downregulated genes we found were HPR, PKLR, PLAU, SOAT2, IYD, OTC and ASGR1. Notably, two metabolic genes, OTC and ASGR1, were previously identified as potential prognostic biomarkers in HCC [63,64]. Several studies have suggested that OTC deficiency in the liver leads to the build-up of ammonia, which causes chronic liver damage, and this is a major risk factor of HCC [65]. Moreover, increased liver fibrosis has been observed in heterologous OTC-KO mice [66]. Additionally, OTC overexpression has been shown to inhibit HCC cell proliferation [63]. Therefore, low OTC expression may enable tumor cells to increase ammonia accumulation, representing a loss of function of the tumor-specific metabolism of OTC. Gu et al. [64] reported that ASGR1 overexpression reduced hepatoma cell migration and invasion by interacting with LASS2. Here, we found that the expression of a serum glycoprotein homeostasis regulator, ASGR1, was downregulated in HNF4A KD cells, suggesting that HNF4A positively regulates ASGR1 expression in HCC cells. Therefore, our result is consistent with the previous report and suggests the role of ASGR1 as a tumor suppressor in HCC [67]. Furthermore, it has been reported that IYD overexpression suppressed Huh7 cell growth by inhibiting glycolysis in HCC cells [68].

Therefore, the downregulation of IYD in HNF4A KD cells is considered as a key driver in HCC malignancy, especially when both HNF1A and HNF4A have loss-of-function activity. However, the contribution of IYD in relation to HNF-family genes to tumorigenesis in the liver has not been investigated yet, and further studies are needed. HPR, PKLR and PLAU have been reported to be overexpressed in breast cancers and esophageal squamous cell carcinoma [69–71]. Conversely, our study showed the downregulation of those genes. It is possible that these genes might be tissue specific, and their downregulation may promote the transition from liver damage to hepatocarcinogenesis and enhance HCC progression in the presence of the loss of function of HNF4A/1A in HCC, but further studies are needed to validate this hypothesis. On the other hand, several genes downregulated in the HNF4A gene network are involved in lipid and cholesterol metabolism, and the downregulation of these genes may promote cancer development. GATA4, APOC3, APOA1 and FOXO1 were found to be downregulated in Huh7 HNF4A KD cells, which were previously reported as cholesterol and lipid metabolism related genes [72–74]. Hepatocyte-specific Gata4-KO mice developed enlarged livers with a proliferative precursor phenotype [75], thus play a role in liver cancer development. HNF4A KD in Huh7 cells reduced SOAT2 mRNA expression. It was previously reported to reduce lipogenesis and de novo cholesterol synthesis in HNF4A KD mice through the inhibition of SOAT2 expression [76]. Based on the overall findings, it is suggested that HNF4A is one of the master regulators of lipid and cholesterol homeostasis, and the disruption of the function of HNF1A caused by mutations may trigger liver cancer development and progression due to the disruption of lipid and cholesterol homeostasis as well as key liver functions such as ammonia and glycoprotein homeostasis. Further in vitro and in vivo studies are required to assess the mutational effect of HNF1A on HCC development.

In conclusion, our study provides new insights into the tumorigenic mechanisms related to HNF1A mutations in the liver. In HCC, our tested mutations in the POU domain of HNF1A that resulted in a loss of function regarding activity in the regulation of the *HNF4A* promoter caused a reduction in HNF4A mRNA expression, with the disruption of lipid metabolism, through the dysregulation of transcriptional networks. Additionally, our findings suggest that HNF1/4A is one of the master regulators of liver cell differentiation and lipid homeostasis and support the idea that any disruption of this transcriptional network may cause liver cancer development and progression.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/genes13030413/s1. Figure S1: The ability of the (A,C) human WT and mutant HNF1A and (B,D) mouse WT and mutant HNF1A to transactivate the target promoter (A,B—*HNF4A*-P2; C,D—*HNF4A*-P2-2200) when overexpressed in HEK293 cells. (E) The ability of the human WT and mutant HNF1A to transactivate the target promoter (*HNF4A*-P2-2200) when overexpressed in Huh7 cells. The cells were co-transfected with the indicated luciferase reporters, and either an empty expression vector (serving as a control) or expression vectors (100 ng) for the indicated HNF1A proteins in 24-well culture plates. The bars indicate the fold activation for HNF1A WT and mutants (vs. control) for target promoters. The corresponding promoter activity is reported as fold activation over control (±SEM, $n = 3$). The data reported represent the averages of three experiments, each performed in duplicate. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Figure S2: HEK293 cells were transfected with expression vectors encoding HNF4A WT or the indicated mutants. (A) Western blot analysis shows that all proteins were similarly expressed. (B) EMSA analysis was used to assess the binding of WT or mutated HNF4A nuclear proteins to a double-stranded oligonucleotide corresponding to the consensus HNF4A-binding elements of the *HNF1A* and *ApoB* promoter region. (C) Structural simulation analysis of the RMSF revealed that mutants have a higher fluctuation rate than WT HNF4A. Figure S3: HNF1A mRNA expression is strongly associated with HNF4A mRNA expression in different cancers. RNA-sequencing data from the TCGA database revealed that HNF4A mRNA expression is highly correlated with HNF1A expression. HNF4A vs. HNF1A. The correlation of gene expression between HNF1A and HNF4A genes was tested using the Spearman's rank correlation test. A positive correlation between HNF1A expression and HNF4A levels was found in CHOL ($r = 0.28$, $p = 0.066$), COAD ($r = 0.52$, $p = 1.1 \times 10^{-23}$), KIRC ($r = 0.64$, $p = 1 \times 10^{-70}$),

KIRP (r = 0.77, $p = 7.3 \times 10^{-65}$), LIHC (r = 0.53, $p = 4.6 \times 10^{-31}$), LUAD (r = 0.56, $p = 4 \times 10^{-45}$), PAAD (r = 0.71, $p = 7.8 \times 10^{-29}$), STAD (r = 0.75, $p = 5.7 \times 10^{-82}$) and READ (r = 0.48, $p = 3.4 \times 10^{-7}$; Supplementary File S1, Figure S3). CHOL—cholangiocarcinoma; COAD—colon adenocarcinoma; KIRC—kidney renal clear cell carcinoma; KIRP—kidney renal papillary cell carcinoma; LIHC—liver hepatocellular carcinoma; LUAD—lung adenocarcinoma; PAAD—pancreatic adenocarcinoma; STAD—stomach adenocarcinoma; READ—rectal adenocarcinoma. Figure S4: Gene ontology analysis of top 10 molecular functions. Table S1: Average values and standard deviations of RMSD, fluctuation, and SASA for each run shown in Figure 3E. The standard deviations are provided in parentheses. The average values and standard errors of the 10 runs are presented in the rightmost column. Table S2: List of primer and siRNA sequences used in the study. Supplementary File S2: Down regulated and upregulated genes.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

DEGs	Differentially expressed genes
DMEM	Dulbecco's modified Eagle's medium
EMSA	Electrophoretic mobility-shift assay
FBS	Fetal bovine serum
GO	Gene ontology
HCC	Hepatocellular carcinoma
HNF1A	Hepatocyte nuclear factor 1A
HNF4A	Hepatocyte nuclear factor 4A
HEK293	Human embryonic kidney cells 293
Huh7	Human hepatoma cell line
IFC	Immunofluorescence
ICGC	International Cancer Genome Consortium
KD	Knockdown
KO	Knockout
KEGG	Kyoto Encyclopedia of Genes and Genomes
MODY3	Maturity-onset diabetes of the young type 3
MD	Molecular dynamics
NGS	Next-generation sequencing
NASH	Non-alcoholic steatohepatitis
Hepa1-6	Non-immunogenic mouse hepatoma cells
NF-κB	Nuclear factor kappa-B
POUh	Pit-Oct-Unc (POU) homeodomain
POUs	Pit-Oct-Unc (POU) specific domain
RMSF	Root-mean-square fluctuation
RMSD	Root-mean-square deviation
TCGA	The Cancer Genome Atlas
LINCS	The Library of Integrated Network-Based Cellular Signatures
VMD	Visual Molecular Dynamics
WB	Western blot
Wnt	Wingless-related integration site

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Supplementary File S1

Table S1: Average values and standard deviations of RMSD, fluctuation, and SASA for each run shown in Figure 3E. The standard deviations are provided in parentheses. The average values and standard error of the 10 runs are presented in the rightmost column

	run1	run2	run3	run4	run5	run6	run7	run8	run9	run10	ave. and std. err. among 10 runs
RMSD of WT protein	2.5(0.1)	3.2(0.6)	2.1(0.5)	2.8(0.4)	1.9(0.2)	2.7(0.4)	1.9(0.2)	1.7(0.3)	1.8(0.2)	1.9(0.2)	2.2(0.2)
RMSD of mutant protein	2.6(0.2)	2.4(0.3)	2.0(0.2)	3.1(0.8)	2.1(0.3)	1.9(0.2)	2.1(0.2)	2.5(0.6)	2.5(0.6)	1.9(0.3)	2.3(0.1)
Fluct. of WT N-ter.	3.4(1.0)	3.7(1.6)	3.9(1.4)	3.8(1.1)	2.8(1.0)	3.6(1.5)	3.6(0.7)	2.6(0.7)	3.1(0.9)	3.9(0.8)	3.4(0.1)
Fluct. of mutant N-ter.	6.8(3.5)	7.1(3.9)	2.8(1.0)	7.7(3.7)	4.4(1.6)	7.3(1.4)	3.7(1.1)	4.8(0.7)	4.4(1.6)	10.3(1.3)	5.9(0.7)
SASA of WT 259th residue	0.3(0.6)	0.4(0.7)	2.1(2.6)	0.8(1.4)	1.8(1.6)	1.1(1.1)	0.8(1.6)	0.4(0.7)	1.3(1.7)	0.5(1.0)	0.9(0.2)
SASA of mutant 259th residue	6.9(6.0)	19.9(14.1)	6.1(4.6)	18.7(18.5)	4.8(4.9)	2.4(3.1)	0.9(1.6)	4.2(3.9)	3.0(2.9)	6.8(7.9)	7.4(2.0)

Table S2: List of Primer and siRNA sequences used in the study

Gene Name	Primer Sequence (5' to 3')	Species	Purpose
HNF1A	5'-gggcggccgcggtttctaagctgagccagc-3'	Mouse	Cloning Primers
HNF1A	5'-ggggtaccttactgggaagaggaggcc-3'	Mouse	Cloning Primers
Y122C HNF1A Mutant	5'-tgttgtgctgctgcaagcacgacttgaccatcttc-3' 5'-gaagatggtcaagtcgtgcttgcagcagcacaaca-3'	Mouse	Mutagenesis
R229Q HNF1A Mutant	5'-tccaccaaggtctcttgctcttccttgctgg-3' 5'-ccagcaaggaagagcaagagaccttggtgga-3'	Mouse	Mutagenesis
V259F HNF1A Mutant	5'-acctccgtgaaaaggttggagcctagccc-3' 5'-gggctaggctccaaccttttcacggaggt-3'	Mouse	Mutagenesis
Y122C HNF1A Mutant	5'-ttgtgctgctgcaggcaggacttgaccatcttc-3' 5'-gaagatggtcaagtcctgcctgcagcagcacaa-3'	Human	Mutagenesis
V259F HNF1A Mutant	5'-cgcacctccgtgaagaggttggagccc-3' 5'-gggctccaacctcttcacggaggtgcg-3'	Human	Mutagenesis
HNF4A P2-2200 (Containing HNF1A Binding site GTTACTCTTTAAC)	5'-ccctaagtgactggttactctttaacgtatccacccacc-3' 5'-ggtgggtggatacgttaaagagtaaccagtcacttaggg-3'		EMSA
Reverse Primer hGH poly A terminator	5'-gcactggggaggggtcacag-3'		Flanking Primers
GAPDH	5'-ggagcgagatccctccaaaat-3' 5'-ggctgttgtcatacttctcatgg-3'	Human	
siRNA HNF4A 1	S- GAC AUU CGG GCG AAG AAG AdTdT A- UCU UCU UCG CCC GAA UGU CdGdC		
siRNA HNF4A 2	S- CAC AAU GCC CAC UCA CdTdT A- GUG AGU GGG CAU UGU GdTdT		

Figure S1. The ability of the (A,C) human WT and mutant HNF1A and (B,D) mouse WT and mutant HNF1A to transactivate the target promoter (A,B—*HNF4A*-P2; C,D—*HNF4A*-P2-2200) when overexpressed in HEK293 cells. (E) The ability of the human WT and mutant HNF1A to transactivate the target promoter (*HNF4A*-P2-2200) when overexpressed in Huh7 cells. The cells were co-transfected with the indicated luciferase reporters, and either an empty expression vector (serving as a control) or expression vectors (100 ng) for the indicated HNF1A proteins in 24-well culture plates. The bars indicate the fold activation for HNF1A WT and mutants (vs. control) for target promoters. The corresponding promoter activity is reported as fold activation over control (±SEM, $n = 3$). The data reported represent the averages of three experiments, each performed in duplicate. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

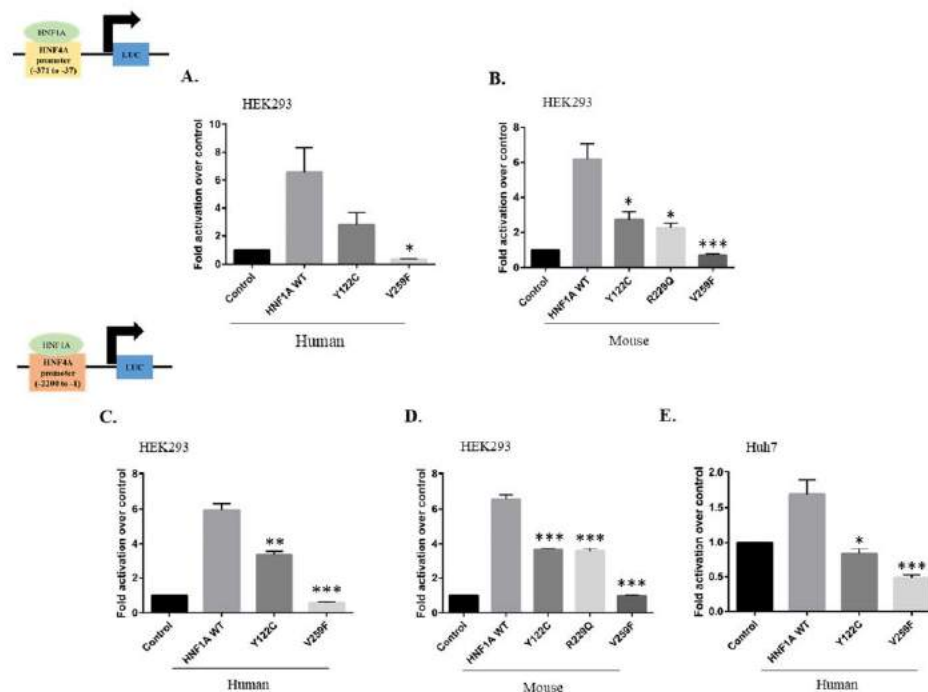


Figure S2. HEK293 cells were transfected with expression vectors encoding HNF4A WT or the indicated mutants. (A) Western blot analysis shows that all proteins were similarly expressed. (B) EMSA analysis was used to assess the binding of WT or mutated HNF4A nuclear proteins to a double-stranded oligonucleotide corresponding to the consensus HNF4A-binding elements of the *HNF1A* and *ApoB* promoter region. (C) Structural simulation analysis of the RMSF revealed that mutants have a higher fluctuation rate than WT HNF4A.

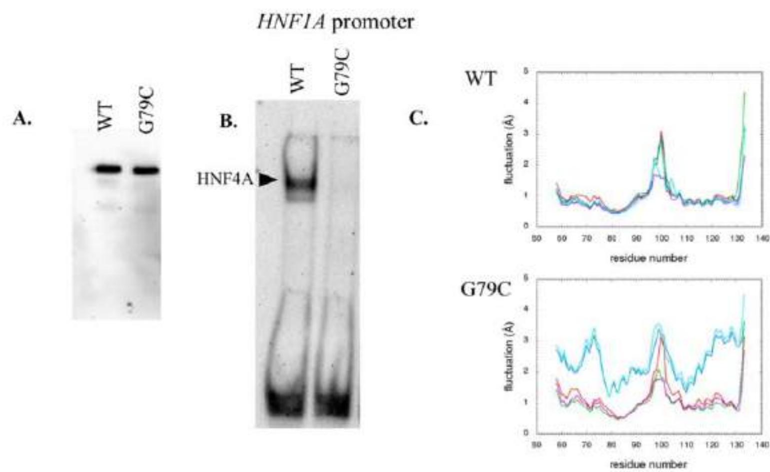


Figure S3. HNF1A mRNA expression is strongly associated with HNF4A mRNA expression in different cancers. RNA-sequencing data from the TCGA database revealed that HNF4A mRNA expression is highly correlated with HNF1A expression. HNF4A vs. HNF1A. The correlation of gene expression between HNF1A and HNF4A genes was tested using the Spearman's rank correlation test. A positive correlation between HNF1A expression and HNF4A levels was found in CHOL ($r = 0.28$, $p = 0.066$), COAD ($r = 0.52$, $p = 1.1 \times 10^{-23}$), KIRC ($r = 0.64$, $p = 1 \times 10^{-70}$), KIRP ($r = 0.77$, $p = 7.3 \times 10^{-65}$), LIHC ($r = 0.53$, $p = 4.6 \times 10^{-31}$), LUAD ($r = 0.56$, $p = 4 \times 10^{-45}$), PAAD ($r = 0.71$, $p = 7.8 \times 10^{-29}$), STAD ($r = 0.75$, $p = 5.7 \times 10^{-82}$) and READ ($r = 0.48$, $p = 3.4 \times 10^{-7}$; Supplementary File 1, Figure S3). CHOL—cholangiocarcinoma; COAD—colon adenocarcinoma; KIRC—kidney renal clear cell carcinoma; KIRP—kidney renal papillary cell carcinoma; LIHC—liver hepatocellular carcinoma; LUAD—lung adenocarcinoma; PAAD—pancreatic adenocarcinoma; STAD—stomach adenocarcinoma; READ—rectal adenocarcinoma.

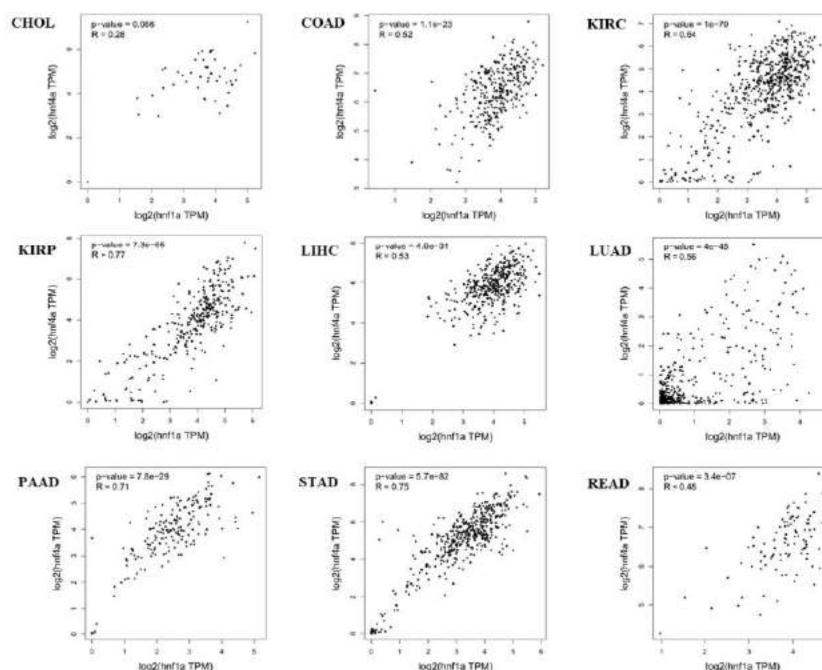
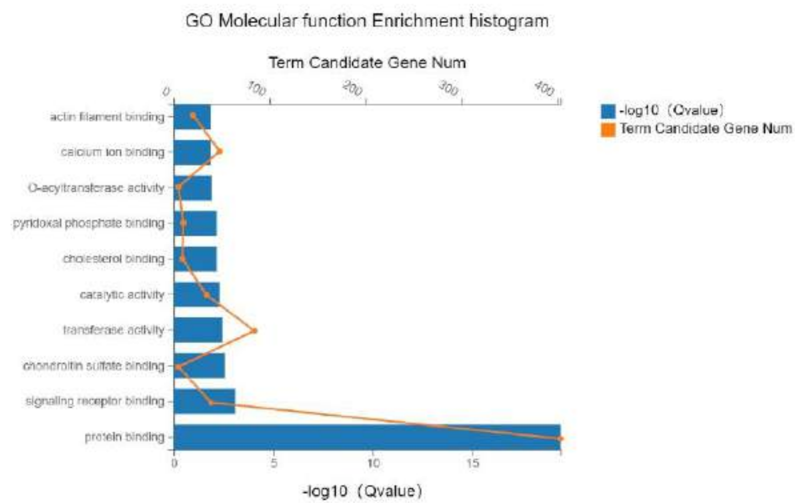


Figure S4. Gene ontology analysis of top 10 molecular functions.



Supplementary file S2

Down-regulated genes					
Gene ID	Gene Symbol	Type	log2 (Knockdown / Control)	Qvalue (Knockdown / Control)	Pvalue(Knockdown / Control)
3250	HPR'	mRNA	-3.9034771	2.40E-06	9.91E-09
5313	PKLR'	mRNA	-3.7552881	1.13E-06	3.73E-09
5328	'PLAU'	mRNA	-3.7505781	1.45E-18	4.27E-22
8435	'SOAT2'	mRNA	-3.5545576	5.17E-09	6.70E-12
389434	IYD'	mRNA	-3.5482524	0.0174538	5.16E-04
5009	OTC'	mRNA	-3.4153927	0.0050453	9.67E-05
432	ASGR1'	mRNA	-3.3391712	3.81E-12	2.25E-15
340152	ZC3H12D'	mRNA	-3.2610388	3.21E-08	6.80E-11
163782	KANK4'	mRNA	-2.9336679	3.17E-27	3.73E-31
653808	'ZG16'	mRNA	-2.8485166	0.0133771	3.53E-04
11251	PTGDR2'	mRNA	-2.7737201	6.44E-06	2.96E-08
8856	'NR1I2'	mRNA	-2.6945835	0.0036094	6.43E-05
6532	'SLC6A4'	mRNA	-2.6069842	8.70E-04	1.06E-05
6291	'SAA4'	mRNA	-2.5919496	0.0057268	1.18E-04
64902	'AGXT2'	mRNA	-2.5660569	3.70E-05	2.12E-07
2326	'FMO1'	mRNA	-2.5409819	0.0382141	0.001511801
283383	'ADGRD1'	mRNA	-2.4014013	3.16E-13	1.30E-16
65266	'WNK4'	mRNA	-2.3623451	1.19E-07	3.00E-10
27141	'CIDEB'	mRNA	-2.3499641	9.74E-11	8.04E-14
1235	'CCR6'	mRNA	-2.3180199	2.77E-05	1.49E-07
3240	'HP'	mRNA	-2.2772957	6.49E-08	1.53E-10
419	'ART3'	mRNA	-2.2318609	0.0056954	1.16E-04
51200	'CPA4'	mRNA	-2.1877601	3.16E-04	2.88E-06
55200	'PLEKHG6'	mRNA	-2.185877	3.58E-07	1.05E-09
64241	'ABCG8'	mRNA	-2.1855841	0.0265976	9.00E-04
51268	'PIPOX'	mRNA	-2.1771348	5.01E-05	2.95E-07
79814	'AGMAT'	mRNA	-2.1501421	2.96E-12	1.57E-15
5948	'RBP2'	mRNA	-2.136829	3.67E-09	4.11E-12
404037	'HAPLN4'	mRNA	-2.0900466	0.0134766	3.59E-04
284417	'TMEM150B'	mRNA	-2.0257204	6.43E-04	7.08E-06
3172	'HNF4A'	mRNA	-2.0000745	3.20E-30	1.89E-34
3957	'LGALS2'	mRNA	-1.9692041	0.0030627	5.20E-05
4622	'MYH4'	mRNA	-1.9500195	0.0045221	8.51E-05
339977	'LRRC66'	mRNA	-1.9462156	0.0011735	1.60E-05
5175	'PECAM1'	mRNA	-1.9381295	1.11E-09	1.12E-12
118788	'PIK3AP1'	mRNA	-1.9124802	1.75E-06	6.61E-09

90288	'EFCAB12'	mRNA	-1.9091804	0.0148009	4.11E-04
388595	'TMEM82'	mRNA	-1.9035157	0.0063646	1.37E-04
1644	'DDC'	mRNA	-1.8829676	8.66E-04	1.05E-05
89870	'TRIM15'	mRNA	-1.8822037	0.0442494	0.001844485
53345	'TM6SF2'	mRNA	-1.8762559	1.09E-06	3.54E-09
2161	'F12'	mRNA	-1.8752349	7.11E-09	1.01E-11
9963	'SLC23A1'	mRNA	-1.8649117	0.0069453	1.53E-04
220001	'VWCE'	mRNA	-1.8458696	5.78E-07	1.77E-09
5002	'SLC22A18'	mRNA	-1.8415084	4.84E-04	4.96E-06
3690	'ITGB3'	mRNA	-1.8159942	0.0145335	3.98E-04
80129	'CCDC170'	mRNA	-1.8084372	0.0026705	4.47E-05
389602	'LOC389602'	mRNA	-1.8030424	0.0252814	8.43E-04
388630	'TRABD2B'	mRNA	-1.7869967	2.34E-06	9.51E-09
83715	'ESPN'	mRNA	-1.7771137	0.0018854	2.88E-05
51703	'ACSL5'	mRNA	-1.7718597	0.0018519	2.76E-05
284111	'SLC13A5'	mRNA	-1.7368981	5.26E-11	4.03E-14
1757	'SARDH'	mRNA	-1.7178069	1.07E-04	7.88E-07
1370	'CPN2'	mRNA	-1.693979	2.48E-08	4.54E-11
9047	'SH2D2A'	mRNA	-1.6855756	0.0348278	0.001324445
2781	'GNAZ'	mRNA	-1.661307	0.0036032	6.37E-05
2786	'GNG4'	mRNA	-1.6508888	3.69E-05	2.09E-07
2165	'F13B'	mRNA	-1.6479683	0.0162655	4.70E-04
1958	'EGR1'	mRNA	-1.616376	0.0016536	2.41E-05
121643	'FOXN4'	mRNA	-1.6056239	0.0166914	4.83E-04
6822	'SULT2A1'	mRNA	-1.5989811	1.83E-08	3.13E-11
55959	'SULF2'	mRNA	-1.5980459	0.0464047	0.001986998
58985	'IL22RA1'	mRNA	-1.5899892	0.0227436	7.36E-04
81855	'SFXN3'	mRNA	-1.5881951	4.06E-08	9.09E-11
84647	'PLA2G12B'	mRNA	-1.5792601	1.94E-06	7.56E-09
11223	'MST1L'	mRNA	-1.5662538	4.42E-09	5.21E-12
5450	'POU2AF1'	mRNA	-1.5662456	0.0100339	2.40E-04
9075	'CLDN2'	mRNA	-1.5049825	0.0063788	1.38E-04
346606	'MOGAT3'	mRNA	-1.504088	0.0438713	0.00182355
9813	'EFCAB14'	mRNA	-1.4984287	5.90E-04	6.34E-06

120425	'JAML'	mRNA	-1.4934587	0.0394858	0.001568215
55231	'CCDC87'	mRNA	-1.4857091	3.69E-04	3.50E-06
9830	'TRIM14'	mRNA	-1.484122	0.0094175	2.23E-04
144717	'PHETA1'	mRNA	-1.4570917	7.50E-07	2.34E-09
345	'APOC3'	mRNA	-1.4564532	8.92E-06	4.21E-08
7464	'CORO2A'	mRNA	-1.4460477	1.56E-10	1.38E-13
5340	'PLG'	mRNA	-1.442006	0.0026212	4.36E-05
92840	'REEP6'	mRNA	-1.4266946	0.0010348	1.32E-05
8694	'DGAT1'	mRNA	-1.420413	0.0111051	2.75E-04
221662	'RBM24'	mRNA	-1.4170612	0.0260454	8.72E-04
3547	'IGSF1'	mRNA	-1.4156529	9.90E-07	3.15E-09
3938	'LCT'	mRNA	-1.4139091	0.044274	0.001853342
374383	'NCR3LG1'	mRNA	-1.4133426	0.0023533	3.82E-05
132158	'GLYCTK'	mRNA	-1.411954	2.03E-04	1.69E-06
81543	'LRRC3'	mRNA	-1.4106117	1.35E-04	1.05E-06
129807	'NEU4'	mRNA	-1.4075984	5.17E-09	6.62E-12
84129	'ACAD11'	mRNA	-1.4054711	0.0016169	2.34E-05
257629	'ANKS4B'	mRNA	-1.403024	0.0100754	2.42E-04
433	'ASGR2'	mRNA	-1.3958336	8.32E-09	1.23E-11
89866	'SEC16B'	mRNA	-1.3945263	0.0046473	8.81E-05
5174	'PDZK1'	mRNA	-1.3943313	0.0283011	9.68E-04
1842	'ECM2'	mRNA	-1.3872556	0.0066747	1.46E-04
130	'ADH6'	mRNA	-1.3825155	3.61E-05	2.02E-07
283375	'SLC39A5'	mRNA	-1.3789913	0.0136252	3.66E-04
114571	'SLC22A9'	mRNA	-1.3777519	0.0024145	3.96E-05
2705	'GJB1'	mRNA	-1.3678085	0.010515	2.58E-04
57205	'ATP10D'	mRNA	-1.3609269	0.0010791	1.43E-05
89782	'LMLN'	mRNA	-1.3494628	0.0213307	6.74E-04
344558	'SH3RF3'	mRNA	-1.3479802	0.0011735	1.59E-05
80830	'APOL6'	mRNA	-1.3473444	0.0062743	1.33E-04
2155	'F7'	mRNA	-1.3430142	1.62E-05	8.04E-08
4143	'MAT1A'	mRNA	-1.3358909	0.0177345	5.30E-04
2168	'FABP1'	mRNA	-1.3313528	0.0154197	4.39E-04
7941	'PLA2G7'	mRNA	-1.325417	4.48E-06	1.95E-08
85358	'SHANK3'	mRNA	-1.3236236	0.0057555	1.18E-04
95	'ACY1'	mRNA	-1.3142304	2.13E-06	8.40E-09
3131	'HLF'	mRNA	-1.3048869	0.0012249	1.69E-05
55244	'SLC47A1'	mRNA	-1.2894315	0.0323666	0.001177418
2053	'EPHX2'	mRNA	-1.2705691	1.43E-06	5.06E-09

6581	'SLC22A3'	mRNA	-1.2541472	0.0053226	1.05E-04
56241	'SUSD2'	mRNA	-1.2456274	7.82E-04	9.22E-06
91860	'CALML4'	mRNA	-1.2430098	9.60E-05	6.49E-07
8309	'ACOX2'	mRNA	-1.2377024	0.0093388	2.20E-04
7456	'WIPF1'	mRNA	-1.2352091	0.0125582	3.22E-04
2593	'GAMT'	mRNA	-1.2298525	0.001054	1.38E-05
189	'AGXT'	mRNA	-1.2209386	0.0265612	8.97E-04
196410	'METTL7B'	mRNA	-1.2170886	1.49E-06	5.55E-09
57462	'MYORG'	mRNA	-1.2167632	0.0266092	9.02E-04
220963	'SLC16A9'	mRNA	-1.2166516	1.81E-04	1.46E-06
2984	'GUCY2C'	mRNA	-1.211114	0.0140399	3.80E-04
9027	'NAT8'	mRNA	-1.2086119	0.0042733	7.94E-05
1230	'CCR1'	mRNA	-1.2058871	0.0464047	0.001989048
285025	'CCDC141'	mRNA	-1.2047261	0.0033066	5.75E-05
18	'ABAT'	mRNA	-1.1741183	9.42E-04	1.15E-05
6299	'SALL1'	mRNA	-1.1671299	0.024709	8.20E-04
7262	'PHLDA2'	mRNA	-1.1643918	0.0024416	4.03E-05
164091	'PAQR7'	mRNA	-1.1545889	0.0134124	3.55E-04
4354	'MPP1'	mRNA	-1.1525753	0.0333776	0.00123781
9942	'XYLB'	mRNA	-1.1405503	0.0018873	2.89E-05
9496	'TBX4'	mRNA	-1.1331865	0.019489	5.91E-04
338094	'FAM151A'	mRNA	-1.1293023	0.0022522	3.61E-05
7049	'TGFBR3'	mRNA	-1.1096249	1.02E-04	7.29E-07
540	'ATP7B'	mRNA	-1.1069972	6.66E-06	3.10E-08
54386	'TERF2IP'	mRNA	-1.1043747	0.0197202	6.08E-04
255043	'TMEM86B'	mRNA	-1.1043697	0.0015959	2.30E-05
4907	'NT5E'	mRNA	-1.0958354	1.26E-05	6.15E-08
340024	'SLC6A19'	mRNA	-1.088345	0.0010678	1.41E-05
4773	'NFATC2'	mRNA	-1.0752765	0.023095	7.52E-04
100507203	'SMLR1'	mRNA	-1.0691347	9.84E-04	1.22E-05
55778	'ZNF839'	mRNA	-1.066199	0.0013296	1.85E-05
8642	'DCHS1'	mRNA	-1.0600152	0.002569	4.26E-05
26230	'TIAM2'	mRNA	-1.0581981	0.0480826	0.002097818
79154	'DHRS11'	mRNA	-1.0540178	0.005092	9.85E-05
56999	'ADAMTS9'	mRNA	-1.0457917	0.0089374	2.10E-04
8492	'PRSS12'	mRNA	-1.0361247	0.0394858	0.001575878
55646	'LYAR'	mRNA	-1.0354859	0.0150172	4.20E-04

4609	'MYC'	mRNA	-1.0348601	0.0373109	0.001469469
2762	'GMDS'	mRNA	-1.0337329	0.0122379	3.11E-04
10849	'CD3EAP'	mRNA	-1.033396	0.0089964	2.12E-04
5618	'PRLR'	mRNA	-1.0261065	1.68E-04	1.34E-06
29965	'CDIP1'	mRNA	-1.0175714	4.79E-04	4.86E-06
435	'ASL'	mRNA	-1.0165248	0.0011735	1.61E-05
1736	'DKC1'	mRNA	-1.015129	0.0094897	2.25E-04
10861	'SLC26A1'	mRNA	-1.0127381	1.03E-08	1.58E-11
282969	'FUOM'	mRNA	-1.0108388	0.0011036	1.48E-05
51022	'GLRX2'	mRNA	-1.0056305	0.0448401	0.001888695
1678	'TIMM8A'	mRNA	-1.0044132	0.0147857	4.09E-04
10062	'NR1H3'	mRNA	-1.0023657	1.75E-05	8.95E-08
392636	'AGMO'	mRNA	-0.9885957	0.0339969	0.001274809
285753	'CEP57L1'	mRNA	-0.9829635	0.0332013	0.001225402
79574	'EPS8L3'	mRNA	-0.9827095	0.0460183	0.001958977
115677	'NOSTRIN'	mRNA	-0.9823653	0.0117046	2.94E-04
10865	'ARID5A'	mRNA	-0.9674533	0.0018532	2.78E-05
26225	'ARL5A'	mRNA	-0.9622417	2.26E-04	1.89E-06
10205	'MPZL2'	mRNA	-0.9590272	0.0191835	5.78E-04
79778	'MICALL2'	mRNA	-0.9517152	0.0064009	1.39E-04
284098	'PIGW'	mRNA	-0.9450869	0.0295407	0.001024005
55612	'FERMT1'	mRNA	-0.9437974	0.0021176	3.32E-05
79762	'C1orf115'	mRNA	-0.9295995	0.0195013	5.97E-04
1384	'CRAT'	mRNA	-0.9247815	6.54E-04	7.33E-06
255027	'MPV17L'	mRNA	-0.924117	0.0394858	0.001576081
55020	'TTC38'	mRNA	-0.9240097	6.05E-06	2.75E-08
124808	'CCDC43'	mRNA	-0.9076979	0.0071713	1.60E-04
2584	'GALK1'	mRNA	-0.9072819	0.0147857	4.07E-04
2859	'GPR35'	mRNA	-0.9041078	0.0101608	2.46E-04
3385	'ICAM3'	mRNA	-0.8990355	0.0108261	2.67E-04
54585	'LZTFL1'	mRNA	-0.8966912	0.0035704	6.29E-05
203427	'SLC25A43'	mRNA	-0.8913788	0.0131272	3.43E-04
80775	'TMEM177'	mRNA	-0.8900951	1.25E-05	6.04E-08
11309	'SLCO2B1'	mRNA	-0.8876507	0.0356968	0.001374329
4485	'MST1'	mRNA	-0.886898	6.17E-05	3.75E-07
309	'ANXA6'	mRNA	-0.8695144	7.99E-04	9.56E-06
60370	'AVPI1'	mRNA	-0.8683083	0.006109	1.28E-04
55092	'TMEM51'	mRNA	-0.8666061	0.0070327	1.55E-04

9245	'GCNT3'	mRNA	-0.8627553	0.0482641	0.002108585
55313	'CPPED1'	mRNA	-0.8571276	6.05E-04	6.57E-06
23566	'LPAR3'	mRNA	-0.8550205	0.0010223	1.29E-05
5244	'ABCB4'	mRNA	-0.8528794	0.0134766	3.59E-04
53841	'CDHR5'	mRNA	-0.8501926	0.0289177	9.94E-04
788	'SLC25A20'	mRNA	-0.8389755	0.0329707	0.001211059
7965	'AIMP2'	mRNA	-0.8374345	1.02E-04	7.20E-07
5805	'PTS'	mRNA	-0.8365671	0.0298572	0.001049759
54458	'PRR13'	mRNA	-0.8356005	2.98E-07	8.26E-10
3628	'INPP1'	mRNA	-0.8347728	3.16E-04	2.88E-06
197	'AHSG'	mRNA	-0.8314062	0.0101552	2.45E-04
9774	'BCLAF1'	mRNA	-0.8212956	0.0096585	2.30E-04
8702	'B4GALT4'	mRNA	-0.8204977	1.68E-05	8.40E-08
81693	'AMN'	mRNA	-0.8185715	0.0483468	0.002115047
23516	'SLC39A14'	mRNA	-0.8131991	0.0018048	2.67E-05
57211	'ADGRG6'	mRNA	-0.8115981	0.0342785	0.001297493
23108	'RAP1GAP2'	mRNA	-0.8088909	0.0221071	7.06E-04
160518	'DENND5B'	mRNA	-0.8042753	1.36E-04	1.06E-06
9965	'FGF19'	mRNA	-0.7956635	0.0033674	5.90E-05
368	'ABCC6'	mRNA	-0.7918507	0.0308902	0.001103648
1962	'EHHADH'	mRNA	-0.7909633	0.0195303	5.99E-04
1662	'DDX10'	mRNA	-0.7893677	0.0342108	0.001289665
90338	'ZNF160'	mRNA	-0.784098	0.0024198	3.98E-05
2628	'GATM'	mRNA	-0.7819733	0.0311497	0.00111662
2118	'ETV4'	mRNA	-0.7788113	6.41E-05	3.93E-07
6615	'SNAI1'	mRNA	-0.7692534	0.0324406	0.001183935
5125	'PCSK5'	mRNA	-0.7562616	0.0197327	6.10E-04
335	'APOA1'	mRNA	-0.7482007	0.0118165	2.98E-04
83862	'TMEM120A'	mRNA	-0.7471407	0.0394858	0.001574955
2805	'GOT1'	mRNA	-0.7438377	0.0115414	2.89E-04
126661	'CCDC163'	mRNA	-0.7415477	0.0333776	0.00123745
873	'CBR1'	mRNA	-0.741542	0.0192913	5.82E-04
4440	'MSI1'	mRNA	-0.7391843	0.0013364	1.87E-05
1519	'CTSO'	mRNA	-0.7383625	0.0302463	0.001069971
388886	'LRRC75B'	mRNA	-0.7377137	0.0484502	0.002122429
90293	'KLHL13'	mRNA	-0.7358269	0.0161566	4.66E-04
131870	'NUDT16'	mRNA	-0.7339403	0.0056954	1.17E-04

65979	'PHACTR4'	mRNA	-0.7304774	0.0176192	5.26E-04
8165	'AKAP1'	mRNA	-0.7293732	1.09E-04	8.13E-07
79962	'DNAJC22'	mRNA	-0.7193045	0.0174538	5.14E-04
5139	'PDE3A'	mRNA	-0.7190712	0.0140399	3.81E-04
64122	'FN3K'	mRNA	-0.7180186	0.0412629	0.001675314
102288414	'C11orf98'	mRNA	-0.7160818	0.0010512	1.35E-05
10244	'RABEPK'	mRNA	-0.7141147	0.023799	7.80E-04
89953	'KLC4'	mRNA	-0.7001012	0.027318	9.29E-04
84365	'NIFK'	mRNA	-0.6989239	5.59E-04	5.94E-06
4547	'MTTP'	mRNA	-0.6959896	0.003293	5.69E-05
661	'POLR3D'	mRNA	-0.6954325	0.0298572	0.001042074
6744	'ITPRID2'	mRNA	-0.6944763	0.0412151	0.001664543
2697	'GJA1'	mRNA	-0.6911058	0.0395563	0.001583559
65263	'PYCR3'	mRNA	-0.6904629	0.0327454	0.001198922
84236	'RHBDD1'	mRNA	-0.6873859	0.0031313	5.34E-05
3615	'IMPDH2'	mRNA	-0.6863116	0.0414313	0.001687934
5862	'RAB2A'	mRNA	-0.6851524	4.01E-04	3.85E-06
4534	'MTM1'	mRNA	-0.6838233	0.0132208	3.48E-04
3156	'HMGCR'	mRNA	-0.6824669	0.0454795	0.001927939
161742	'SPRED1'	mRNA	-0.6734395	0.0400259	0.001609438
119559	'SFXN4'	mRNA	-0.6697577	0.0311361	0.001114298
10723	'SLC12A7'	mRNA	-0.6643375	0.0221772	7.11E-04
63910	'SLC17A9'	mRNA	-0.6642191	0.0154767	4.43E-04
5833	'PCYT2'	mRNA	-0.6619406	0.0055013	1.10E-04
11264	'PXMP4'	mRNA	-0.6553191	0.0100754	2.42E-04
51097	'SCCPDH'	mRNA	-0.6548995	0.0056756	1.14E-04
54534	'MRPL50'	mRNA	-0.652843	0.02345	7.66E-04
5831	'PYCR1'	mRNA	-0.6473916	0.0147857	4.09E-04
2582	'GALE'	mRNA	-0.6458611	0.0096585	2.31E-04
9701	'PPP6R2'	mRNA	-0.6457142	0.0422164	0.001738611
65260	'COA7'	mRNA	-0.6434466	0.0152394	4.30E-04
7005	'TEAD3'	mRNA	-0.6428985	0.0298572	0.001050927
26872	'STEAP1'	mRNA	-0.6421919	0.0362954	0.001407563
10788	'IQGAP2'	mRNA	-0.6352299	0.0422164	0.00174199
2308	'FOXO1'	mRNA	-0.6318155	0.0199566	6.22E-04
57062	'DDX24'	mRNA	-0.6314674	0.0198392	6.18E-04
11046	'SLC35D2'	mRNA	-0.6295698	0.023095	7.51E-04
51061	'TXNDC11'	mRNA	-0.6290933	0.0023896	3.90E-05
162967	'ZNF320'	mRNA	-0.628893	0.0114328	2.84E-04

5106	'PCK2'	mRNA	-0.6282894	0.003641	6.53E-05
26063	'DECR2'	mRNA	-0.6257863	0.006006	1.25E-04
1445	'CSK'	mRNA	-0.6230104	0.0218986	6.96E-04
51114	'ZDHHC9'	mRNA	-0.6201959	0.0220346	7.02E-04
84154	'RPF2'	mRNA	-0.6169882	0.0150172	4.21E-04
81932	'HDHD3'	mRNA	-0.6108646	0.0087145	2.03E-04
8797	'TNFRSF10A'	mRNA	-0.6097412	0.0448401	0.001892074
7726	'TRIM26'	mRNA	-0.6095539	7.86E-04	9.31E-06
344	'APOC2'	mRNA	-0.6069418	0.0055807	1.12E-04
6927	'HNF1A'	mRNA	-0.6056608	0.0351287	0.001344729
84769	'MPV17L2'	mRNA	-0.6027055	0.0367849	0.00143574
53838	'C11orf24'	mRNA	-0.6026359	4.43E-04	4.41E-06
3291	'HSD11B2'	mRNA	-0.5986268	0.0262044	8.82E-04
84067	'FAM160A2'	mRNA	-0.5940285	0.0241904	7.97E-04
801	'CALM1'	mRNA	-0.5916922	0.0422164	0.00173522
29841	'GRHL1'	mRNA	-0.5902431	0.0479062	0.002084475
56181	'MTFR1L'	mRNA	-0.5894395	0.0412629	0.001676205
6383	'SDC2'	mRNA	-0.5870799	0.0149593	4.16E-04
23239	'PHLPP1'	mRNA	-0.5852616	0.0453806	0.001921069
19	'ABCA1'	mRNA	-0.5823688	0.0122146	3.09E-04
80339	'PNPLA3'	mRNA	-0.5766583	0.0240466	7.90E-04
4600	'MX2'	mRNA	-0.569128	0.0494689	0.002172885
7804	'LRP8'	mRNA	-0.5671777	0.0153146	4.34E-04
2626	'GATA4'	mRNA	-0.5601408	0.0460183	0.001959783
10897	'YIF1A'	mRNA	-0.5584819	0.0445767	0.001871271
6945	'MLX'	mRNA	-0.5546654	0.0055807	1.12E-04
22872	'SEC31A'	mRNA	-0.546071	0.0086183	1.99E-04
7873	'MANF'	mRNA	-0.544828	0.0315022	0.001138545
8140	'SLC7A5'	mRNA	-0.5442185	0.0342108	0.001290897
10813	'UTP14A'	mRNA	-0.5428284	0.0215709	6.84E-04
54552	'GNL3L'	mRNA	-0.5409045	0.0160775	4.63E-04
10994	'ILVBL'	mRNA	-0.5274957	0.0317248	0.001148459
84864	'RIOX2'	mRNA	-0.5243461	0.0145335	3.98E-04
4258	'MGST2'	mRNA	-0.517341	0.0221772	7.11E-04
85403	'EAF1'	mRNA	-0.5020202	0.0267772	9.09E-04
471	'ATIC'	mRNA	-0.4981331	0.0334812	0.001247793
9883	'POM121'	mRNA	-0.4959369	0.0194994	5.93E-04
64744	'SMAP2'	mRNA	-0.4944356	0.0197202	6.08E-04
8501	'SLC43A1'	mRNA	-0.4851068	0.0131179	3.42E-04
10456	'HAX1'	mRNA	-0.4816788	0.0402482	0.00162075
4224	'MEP1A'	mRNA	-0.4717552	0.0422164	0.001742318

9868	'TOMM70'	mRNA	-0.4684956	0.0345234	0.001310836
23135	'KDM6B'	mRNA	-0.4632681	0.044582	0.001874122
23481	'PES1'	mRNA	-0.4568675	0.0220346	7.03E-04
23464	'GCAT'	mRNA	-0.4568662	0.0444482	0.001863256
80790	'CMIP'	mRNA	-0.4553084	0.0497754	0.002192219
10131	'TRAP1'	mRNA	-0.4544048	0.0195013	5.96E-04
83606	'GUCD1'	mRNA	-0.4533639	0.0323182	0.001173752
1314	'COPA'	mRNA	-0.4502521	0.0383202	0.001518259
9761	'MLEC'	mRNA	-0.4308138	0.0499303	0.002201985
3949	'LDLR'	mRNA	-0.4305502	0.0396077	0.00158795

Up-regulated genes					
Gene ID	Gene Symbol	Type	log2 (Knockdown / Control)	Qvalue (Knockdown / Control)	Pvalue(Knockdown / Control)
284040	CDRT4'	mRNA	19.65262	3.27E-04	3.02E-06
100534012	TNFAIP8L2-SCNM1'	mRNA	6.946153	0.00106	1.39E-05
644634	LOC644634'	lncRNA	6.639181	0.019489	5.90E-04
3764	KCNJ8'	mRNA	3.433539	4.30E-06	1.82E-08
202151	RANBP3L'	mRNA	3.024791	0.005323	1.05E-04
131368	'ZPLD1'	mRNA	2.96741	4.84E-04	4.97E-06
165679	'SPTSSB'	mRNA	2.866375	2.27E-06	9.12E-09
286204	CRB2'	mRNA	2.627778	2.33E-17	8.23E-21
107987464	LOC107987464'	mRNA	2.579538	0.035129	0.001346
57575	PCDH10'	mRNA	2.534078	0.001538	2.20E-05
79642	ARSJ'	mRNA	2.487224	7.09E-04	8.01E-06
146754	'DNAH2'	mRNA	2.408132	0.043475	0.001799
90113	'VWA5B2'	mRNA	2.39478	9.87E-04	1.24E-05
25837	'RAB26'	mRNA	2.377148	0.046206	0.001972
114131	'UCN3'	mRNA	2.304813	0.008016	1.81E-04
9148	NEURL1'	mRNA	2.214876	0.005183	1.01E-04
120114	'FAT3'	mRNA	2.200237	0.00192	2.98E-05
91807	'MYLK3'	mRNA	2.181326	7.70E-04	9.03E-06
54567	'DLL4'	mRNA	2.176211	2.66E-05	1.41E-07
4017	'LOXL2'	mRNA	2.148418	7.27E-05	4.76E-07
165082	'ADGRF3'	mRNA	2.11258	0.002671	4.46E-05
5159	'PDGFRB'	mRNA	2.069511	0.001233	1.71E-05
64221	'ROBO3'	mRNA	2.057217	0.030863	0.001097
3760	'KCNJ3'	mRNA	2.032048	0.001911	2.94E-05
11238	'CA5B'	mRNA	2.023555	7.54E-04	8.71E-06

114088	'TRIM9'	mRNA	1.992784	4.55E-04	4.56E-06
6275	'S100A4'	mRNA	1.979364	0.002118	3.33E-05
83959	'SLC4A11'	mRNA	1.900733	6.02E-05	3.62E-07
80164	'PRR36'	mRNA	1.871431	0.001054	1.38E-05
1288	'COL4A6'	mRNA	1.830467	0.001629	2.36E-05
2302	'FOXJ1'	mRNA	1.826104	0.012812	3.32E-04
5493	'PPL'	mRNA	1.824621	1.43E-07	3.87E-10
7289	'TULP3'	mRNA	1.822181	4.70E-19	1.11E-22
7092	'TLL1'	mRNA	1.782313	0.012563	3.23E-04
23362	'PSD3'	mRNA	1.762635	1.95E-21	3.45E-25
338557	'FFAR4'	mRNA	1.760004	0.015405	4.38E-04
55089	'SLC38A4'	mRNA	1.747312	3.14E-08	6.30E-11
84866	'TMEM25'	mRNA	1.716933	4.03E-04	3.94E-06
6866	'TAC3'	mRNA	1.71347	0.014801	4.10E-04
9537	'TP53I11'	mRNA	1.70931	3.70E-05	2.14E-07
3109	'HLA-DMB'	mRNA	1.68618	0.002021	3.16E-05
4320	'MMP11'	mRNA	1.671494	0.008364	1.91E-04
2535	'FZD2'	mRNA	1.667546	0.004606	8.69E-05
389792	'IER5L'	mRNA	1.646151	7.56E-04	8.78E-06
10417	'SPON2'	mRNA	1.623415	0.006006	1.25E-04
54855	'TENT5C'	mRNA	1.617644	0.001912	2.95E-05
222663	'SCUBE3'	mRNA	1.598525	1.47E-06	5.30E-09
154743	'BMT2'	mRNA	1.591777	2.55E-08	4.82E-11
5325	'PLAGL1'	mRNA	1.587841	1.02E-05	4.85E-08
397	'ARHGDIB'	mRNA	1.585587	0.002709	4.57E-05
7043	'TGFB3'	mRNA	1.573469	0.022206	7.14E-04
28968	'SLC6A16'	mRNA	1.563849	0.045027	0.001903
140576	'S100A16'	mRNA	1.553981	9.60E-05	6.51E-07
3351	'HTR1B'	mRNA	1.552028	7.15E-04	8.14E-06
79180	'EFHD2'	mRNA	1.550314	6.40E-12	4.15E-15
55061	'SUSD4'	mRNA	1.547774	0.00239	3.89E-05
3222	'HOXC5'	mRNA	1.544805	0.001225	1.68E-05
5420	'PODXL'	mRNA	1.537881	1.02E-04	7.16E-07
5074	'PAWR'	mRNA	1.534393	3.14E-08	6.47E-11
29969	'MDFIC'	mRNA	1.52907	9.44E-08	2.34E-10
4616	'GADD45B'	mRNA	1.528167	3.40E-07	9.61E-10

57538	'ALPK3'	mRNA	1.51455	2.46E-09	2.61E-12
440279	'UNC13C'	mRNA	1.514233	0.010585	2.60E-04
2104	'ESRRG'	mRNA	1.513646	0.001784	2.63E-05
1831	'TSC22D3'	mRNA	1.512933	0.001113	1.50E-05
2982	'GUCY1A1'	mRNA	1.512496	0.022492	7.25E-04
11275	'KLHL2'	mRNA	1.505896	2.29E-05	1.19E-07
6696	'SPP1'	mRNA	1.505615	0.012812	3.32E-04
22898	'DENND3'	mRNA	1.490311	3.48E-04	3.26E-06
200810	'ALG1L'	mRNA	1.488536	0.034433	0.001305
642515	'PRRT1B'	mRNA	1.473402	0.001027	1.30E-05
728568	'C12orf73'	mRNA	1.46216	7.34E-04	8.43E-06
29942	'PURG'	mRNA	1.444952	0.010203	2.49E-04
3696	'ITGB8'	mRNA	1.441609	0.001054	1.37E-05
116372	'LYPD1'	mRNA	1.412314	0.017454	5.19E-04
644538	'SMIM10'	mRNA	1.398634	0.008628	2.00E-04
23705	'CADM1'	mRNA	1.395025	0.001781	2.61E-05
84466	'MEGF10'	mRNA	1.369704	5.92E-10	5.59E-13
11010	'GLIPR1'	mRNA	1.369608	1.49E-06	5.51E-09
1303	'COL12A1'	mRNA	1.364389	3.65E-11	2.58E-14
3491	'CYR61'	mRNA	1.357823	1.80E-06	6.89E-09
55214	'P3H2'	mRNA	1.349537	1.02E-04	7.16E-07
55203	'LGI2'	mRNA	1.347293	1.20E-07	3.12E-10
1368	'CPM'	mRNA	1.345496	1.17E-08	1.86E-11
222546	'RFX6'	mRNA	1.345119	4.95E-06	2.22E-08
28962	'OSTM1'	mRNA	1.337683	6.35E-13	3.00E-16
999	'CDH1'	mRNA	1.331488	0.003825	6.90E-05
3797	'KIF3C'	mRNA	1.323747	6.61E-05	4.12E-07
7484	'WNT9B'	mRNA	1.320132	0.029857	0.001049
4237	'MFAP2'	mRNA	1.319497	0.001173	1.60E-05
23555	'TSPAN15'	mRNA	1.318764	2.84E-05	1.54E-07
388228	'SBK1'	mRNA	1.312807	7.00E-05	4.50E-07
10382	'TUBB4A'	mRNA	1.309851	0.004031	7.44E-05
107985770	'LOC107985770'	lncRNA	1.305445	0.037013	0.001452
389332	'SMIM32'	mRNA	1.303606	0.00335	5.85E-05
167227	'DCP2'	mRNA	1.300208	1.36E-08	2.24E-11
8912	'CACNA1H'	mRNA	1.299762	0.015239	4.29E-04
85360	'SYDE1'	mRNA	1.296304	3.56E-07	1.03E-09

9516	'LITAF'	mRNA	1.285854	0.002683	4.51E-05
8654	'PDE5A'	mRNA	1.272609	1.05E-04	7.71E-07
26960	'NBEA'	mRNA	1.269246	0.040781	0.001645
23529	'CLCF1'	mRNA	1.266295	0.012801	3.31E-04
5209	'PFKFB3'	mRNA	1.262287	1.35E-06	4.61E-09
6624	'FSCN1'	mRNA	1.262107	0.006558	1.43E-04
100271849	'MEF2B'	mRNA	1.260262	0.012668	3.26E-04
29799	'YPEL1'	mRNA	1.258983	0.02989	0.001054
11167	'FSTL1'	mRNA	1.255431	0.017454	5.15E-04
2817	'GPC1'	mRNA	1.245193	0.038147	0.001507
10769	'PLK2'	mRNA	1.243293	7.37E-08	1.78E-10
55529	'PIP4P2'	mRNA	1.242229	0.017454	5.19E-04
57094	'CPA6'	mRNA	1.239227	0.010203	2.49E-04
123228	'SENP8'	mRNA	1.224931	0.030385	0.001078
84456	'L3MBTL3'	mRNA	1.219788	0.00381	6.85E-05
90427	'BMF'	mRNA	1.219635	3.16E-04	2.85E-06
10479	'SLC9A6'	mRNA	1.21807	6.80E-05	4.33E-07
27124	'INPP5J'	mRNA	1.217054	3.14E-08	6.12E-11
132720	'FAM241A'	mRNA	1.211993	8.13E-04	9.78E-06
130271	'PLEKHH2'	mRNA	1.210101	1.05E-04	7.62E-07
146760	'RTN4RL1'	mRNA	1.208484	0.017454	5.13E-04
57393	'CLTRN'	mRNA	1.207634	4.31E-06	1.85E-08
4688	'NCF2'	mRNA	1.191185	0.047886	0.002078
51696	'HECA'	mRNA	1.188985	3.42E-04	3.19E-06
84662	'GLIS2'	mRNA	1.187451	1.98E-04	1.63E-06
8828	'NRP2'	mRNA	1.184404	3.16E-04	2.86E-06
147463	'ANKRD29'	mRNA	1.182401	0.035594	0.001368
3400	'ID4'	mRNA	1.180366	0.005045	9.70E-05
115294	'PCMTD1'	mRNA	1.179921	0.008206	1.86E-04
283987	'HID1'	mRNA	1.176298	3.34E-05	1.83E-07
83716	'CRISPLD2'	mRNA	1.168301	0.043629	0.001811
780	'DDR1'	mRNA	1.167102	6.61E-05	4.13E-07
107985082	'LOC107985082'	mRNA	1.1592	0.001513	2.14E-05
301	'ANXA1'	mRNA	1.15552	1.33E-04	1.02E-06
255743	'NPNT'	mRNA	1.152153	0.046825	0.002015
824	'CAPN2'	mRNA	1.134627	3.85E-07	1.16E-09
283229	'CRACR2B'	mRNA	1.129292	0.032881	0.001206

113220	'KIF12'	mRNA	1.129131	1.39E-07	3.69E-10
223117	'SEMA3D'	mRNA	1.125292	0.034146	0.001284
6337	'SCNN1A'	mRNA	1.111523	0.011105	2.76E-04
55652	'SLC48A1'	mRNA	1.111484	0.002127	3.36E-05
2151	'F2RL2'	mRNA	1.103926	9.59E-04	1.18E-05
80854	'SETD7'	mRNA	1.103702	9.99E-05	6.89E-07
5865	'RAB3B'	mRNA	1.100222	3.19E-06	1.34E-08
3092	'HIP1'	mRNA	1.09474	2.42E-08	4.28E-11
23452	'ANGPTL2'	mRNA	1.092222	0.001853	2.79E-05
9246	'UBE2L6'	mRNA	1.088553	0.003915	7.09E-05
139818	'DOCK11'	mRNA	1.088013	4.95E-04	5.17E-06
84959	'UBASH3B'	mRNA	1.087912	0.006558	1.43E-04
30061	'SLC40A1'	mRNA	1.087423	0.001773	2.59E-05
7089	'TLE2'	mRNA	1.079731	0.003267	5.62E-05
7336	'UBE2V2'	mRNA	1.075578	1.27E-06	4.28E-09
285513	'GPRIN3'	mRNA	1.075543	0.029233	0.00101
3832	'KIF11'	mRNA	1.075421	1.37E-06	4.77E-09
1466	'CSRP2'	mRNA	1.069654	0.04781	0.002072
635	'BHMT'	mRNA	1.068775	0.030246	0.00107
285368	'PRRT3'	mRNA	1.067646	0.016891	4.93E-04
255403	'ZNF718'	mRNA	1.067592	0.033009	0.001214
5087	'PBX1'	mRNA	1.066793	4.79E-08	1.10E-10
83787	'ARMC10'	mRNA	1.065841	4.95E-04	5.14E-06
8875	'VNN2'	mRNA	1.064575	2.75E-04	2.41E-06
58472	'SQOR'	mRNA	1.059583	3.21E-04	2.95E-06
135112	'NCOA7'	mRNA	1.058672	7.87E-05	5.20E-07
23432	'GPR161'	mRNA	1.058008	0.005814	1.20E-04
23271	'CAMSAP2'	mRNA	1.057061	5.83E-09	7.91E-12
858	'CAV2'	mRNA	1.052272	1.14E-04	8.50E-07
91283	'MSANTD3'	mRNA	1.050852	7.27E-05	4.75E-07
85464	'SSH2'	mRNA	1.049771	3.93E-08	8.56E-11
54809	'SAMD9'	mRNA	1.049632	0.009513	2.26E-04
127435	'PODN'	mRNA	1.048122	0.036095	0.001394
8434	'RECK'	mRNA	1.041208	0.014598	4.00E-04
5412	'UBL3'	mRNA	1.038992	2.29E-04	1.96E-06
8869	'ST3GAL5'	mRNA	1.034255	2.93E-04	2.59E-06
8463	'TEAD2'	mRNA	1.034052	2.39E-04	2.06E-06
6304	'SATB1'	mRNA	1.027316	3.88E-04	3.71E-06
54205	'CYCS'	mRNA	1.026636	2.01E-04	1.66E-06

9021	'SOCS3'	mRNA	1.025881	0.013143	3.45E-04
205428	'C3orf58'	mRNA	1.011171	9.60E-05	6.45E-07
84804	'MFSD9'	mRNA	1.007597	1.25E-04	9.41E-07
3726	'JUNB'	mRNA	0.9992	0.007953	1.79E-04
7373	'COL14A1'	mRNA	0.997176	0.005313	1.04E-04
92421	'CHMP4C'	mRNA	0.99714	0.001837	2.73E-05
83593	'RASSF5'	mRNA	0.995137	0.015239	4.30E-04
8905	'AP1S2'	mRNA	0.994428	0.002252	3.60E-05
55205	'ZNF532'	mRNA	0.993878	3.16E-04	2.89E-06
3306	'HSPA2'	mRNA	0.993766	0.002153	3.42E-05
4602	'MYB'	mRNA	0.992973	0.004338	8.11E-05
56967	'C14orf132'	mRNA	0.990657	6.53E-04	7.28E-06
83543	'AIF1L'	mRNA	0.990448	0.028368	9.72E-04
84102	'SLC41A2'	mRNA	0.988569	0.013593	3.64E-04
151887	'CCDC80'	mRNA	0.987792	0.004292	8.00E-05
4148	'MATN3'	mRNA	0.987441	0.049775	0.002192
25978	'CHMP2B'	mRNA	0.986925	0.001976	3.08E-05
93145	'OLFM2'	mRNA	0.98627	7.25E-04	8.30E-06
7162	'TPBG'	mRNA	0.983242	0.046657	0.002005
4240	'MFGE8'	mRNA	0.982896	4.63E-04	4.67E-06
1123	'CHN1'	mRNA	0.978825	0.029032	1.00E-03
152007	'GLIPR2'	mRNA	0.975714	0.005695	1.16E-04
100505385	'IQCJ-SCHIP1'	mRNA	0.970675	0.00108	1.44E-05
4355	'MPP2'	mRNA	0.969116	0.006945	1.53E-04
373	'TRIM23'	mRNA	0.96463	0.034146	0.001283
253558	'LCLAT1'	mRNA	0.962545	5.28E-04	5.54E-06
22795	'NID2'	mRNA	0.959639	0.03722	0.001464
414149	'ACBD7'	mRNA	0.959023	0.039556	0.001582
5357	'PLS1'	mRNA	0.95461	0.001536	2.19E-05
55284	'UBE2W'	mRNA	0.952644	0.003609	6.45E-05
26047	'CNTNAP2'	mRNA	0.951718	0.013412	3.56E-04
51232	'CRIM1'	mRNA	0.950964	0.008239	1.88E-04
51157	'ZNF580'	mRNA	0.948608	0.035129	0.001344
7320	'UBE2B'	mRNA	0.946751	4.49E-06	1.99E-08
4879	'NPPB'	mRNA	0.942635	0.001521	2.16E-05
25946	'ZNF385A'	mRNA	0.941595	2.28E-04	1.94E-06

59277	'NTN4'	mRNA	0.941033	0.035979	0.001387
126969	'SLC44A3'	mRNA	0.939631	0.006675	1.46E-04
84918	'LRP11'	mRNA	0.93532	1.72E-05	8.74E-08
27250	'PDCD4'	mRNA	0.934006	0.011541	2.89E-04
25842	'ASF1A'	mRNA	0.93178	2.47E-04	2.14E-06
6604	'SMARCD3'	mRNA	0.931359	0.008429	1.93E-04
170954	'PPP1R18'	mRNA	0.927765	0.006109	1.28E-04
84451	'MAP3K21'	mRNA	0.927152	0.029541	0.001026
5922	'RASA2'	mRNA	0.925315	2.75E-04	2.41E-06
5413	'SEPT5'	mRNA	0.923283	0.001864	2.81E-05
6690	'SPINK1'	mRNA	0.920798	0.021001	6.61E-04
149420	'PDIK1L'	mRNA	0.920363	0.012419	3.17E-04
258010	'SVIP'	mRNA	0.919878	0.016891	4.92E-04
51099	'ABHD5'	mRNA	0.917435	0.001045	1.34E-05
11247	'NXPH4'	mRNA	0.917193	0.003938	7.15E-05
2057	'EPOR'	mRNA	0.915942	0.001879	2.86E-05
3880	'KRT19'	mRNA	0.91473	0.04414	0.001837
9262	'STK17B'	mRNA	0.913228	0.00401	7.33E-05
4900	'NRGN'	mRNA	0.911936	0.041469	0.001692
3911	'LAMA5'	mRNA	0.910807	0.008747	2.04E-04
83999	'KREMEN1'	mRNA	0.90928	0.005005	9.56E-05
8322	'FZD4'	mRNA	0.905397	7.99E-04	9.56E-06
11098	'PRSS23'	mRNA	0.904018	1.36E-04	1.07E-06
6102	'RP2'	mRNA	0.903987	0.021571	6.83E-04
3248	'HPGD'	mRNA	0.903972	0.006268	1.33E-04
55074	'OXR1'	mRNA	0.903694	0.005695	1.15E-04
8971	'H1FX'	mRNA	0.896632	3.58E-04	3.38E-06
11221	'DUSP10'	mRNA	0.894304	9.87E-04	1.23E-05
57619	'SHROOM3'	mRNA	0.889272	2.29E-05	1.20E-07
23594	'ORC6'	mRNA	0.877362	0.006323	1.35E-04
376267	'RAB15'	mRNA	0.876771	0.005343	1.06E-04
2674	'GFRA1'	mRNA	0.87388	0.047726	0.002065
11237	'RNF24'	mRNA	0.873481	5.81E-05	3.46E-07
857	'CAV1'	mRNA	0.871674	0.003609	6.40E-05
283209	'PGM2L1'	mRNA	0.863531	0.034278	0.001297
1809	'DPYSL3'	mRNA	0.860572	0.047252	0.002039
84706	'GPT2'	mRNA	0.860401	0.008876	2.08E-04
23589	'CARHSP1'	mRNA	0.859771	0.006274	1.34E-04

9353	'SLIT2'	mRNA	0.859429	0.005939	1.23E-04
22881	'ANKRD6'	mRNA	0.856351	0.039486	0.001572
4739	'NEDD9'	mRNA	0.854375	0.047229	0.002036
5825	'ABCD3'	mRNA	0.852286	1.05E-04	7.60E-07
83660	'TLN2'	mRNA	0.850375	0.005096	9.89E-05
726	'CAPN5'	mRNA	0.845694	1.48E-04	1.17E-06
4683	'NBN'	mRNA	0.842986	7.70E-04	9.02E-06
55974	'SLC50A1'	mRNA	0.840858	6.66E-05	4.20E-07
81573	'ANKRD13C'	mRNA	0.84059	2.28E-04	1.92E-06
1633	'DCK'	mRNA	0.839496	0.022492	7.27E-04
199731	'CADM4'	mRNA	0.838926	0.032554	0.00119
2743	'GLRB'	mRNA	0.831418	0.023261	7.58E-04
23075	'SWAP70'	mRNA	0.828906	0.025281	8.41E-04
117177	'RAB3IP'	mRNA	0.828396	9.82E-05	6.72E-07
10891	'PPARGC1A'	mRNA	0.822673	0.005577	1.11E-04
375061	'FAM89A'	mRNA	0.815142	0.002272	3.66E-05
5747	'PTK2'	mRNA	0.812835	0.003172	5.44E-05
51222	'ZNF219'	mRNA	0.812653	0.039962	0.001605
113263	'GLCCI1'	mRNA	0.812346	0.001054	1.36E-05
57544	'TXNDC16'	mRNA	0.810966	0.041247	0.001671
162239	'ZFP1'	mRNA	0.810361	0.007171	1.60E-04
65062	'TMEM237'	mRNA	0.803259	0.005055	9.75E-05
10079	'ATP9A'	mRNA	0.802972	0.010279	2.51E-04
1946	'EFNA5'	mRNA	0.801981	0.031168	0.00112
25959	'KANK2'	mRNA	0.801893	0.022492	7.26E-04
9294	'S1PR2'	mRNA	0.800668	0.00134	1.88E-05
24145	'PANX1'	mRNA	0.800375	5.78E-04	6.16E-06
9828	'ARHGEF17'	mRNA	0.799675	0.007363	1.65E-04
84909	'C9orf3'	mRNA	0.799671	0.003526	6.20E-05
114569	'MAL2'	mRNA	0.798192	0.020412	6.39E-04
27122	'DKK3'	mRNA	0.798124	1.96E-04	1.59E-06
23012	'STK38L'	mRNA	0.793853	0.004113	7.62E-05
89958	'SAPCD2'	mRNA	0.788636	3.43E-05	1.90E-07
57561	'ARRDC3'	mRNA	0.787373	0.004838	9.21E-05
83699	'SH3BGRL2'	mRNA	0.787217	0.030385	0.001078
9265	'CYTH3'	mRNA	0.784577	0.004027	7.41E-05
729438	'CASTOR2'	mRNA	0.784433	1.77E-04	1.42E-06
51313	'FAM198B'	mRNA	0.782557	0.024087	7.92E-04
64359	'NXN'	mRNA	0.778855	0.033481	0.00125
1843	'DUSP1'	mRNA	0.773049	0.005343	1.06E-04

83666	'PARP9'	mRNA	0.772342	0.005196	1.02E-04
6777	'STAT5B'	mRNA	0.770169	0.017933	5.37E-04
2888	'GRB14'	mRNA	0.766561	0.046018	0.001962
5324	'PLAG1'	mRNA	0.76604	0.035129	0.001344
3993	'LLGL2'	mRNA	0.765717	4.03E-04	3.97E-06
1456	'CSNK1G3'	mRNA	0.762854	0.014703	4.04E-04
6525	'SMTN'	mRNA	0.760287	0.00635	1.36E-04
9194	'SLC16A7'	mRNA	0.760283	0.031457	0.001135
7286	'TUFT1'	mRNA	0.759965	0.004647	8.82E-05
710	'SERPING1'	mRNA	0.756654	0.003299	5.72E-05
5287	'PIK3C2B'	mRNA	0.755369	6.05E-04	6.54E-06
9423	'NTN1'	mRNA	0.754686	0.015017	4.20E-04
3223	'HOXC6'	mRNA	0.752772	0.028463	9.77E-04
51465	'UBE2J1'	mRNA	0.7499	4.03E-04	3.92E-06
222962	'SLC29A4'	mRNA	0.746609	0.003962	7.22E-05
10732	'TCFL5'	mRNA	0.74637	0.006371	1.37E-04
55120	'FANCL'	mRNA	0.746033	0.015306	4.33E-04
92922	'CCDC102A'	mRNA	0.743562	0.019501	5.95E-04
4090	'SMAD5'	mRNA	0.743094	0.017454	5.20E-04
144100	'PLEKHA7'	mRNA	0.74305	0.02615	8.79E-04
64847	'SPATA20'	mRNA	0.741184	0.044274	0.00185
23548	'TTC33'	mRNA	0.74067	0.006141	1.29E-04
64750	'SMURF2'	mRNA	0.739775	0.027416	9.34E-04
79647	'AKIRIN1'	mRNA	0.739642	4.86E-04	5.02E-06
3613	'IMPA2'	mRNA	0.739636	4.03E-04	3.93E-06
3556	'IL1RAP'	mRNA	0.738573	6.53E-04	7.27E-06
54625	'PARP14'	mRNA	0.738246	0.013143	3.45E-04
135114	'HINT3'	mRNA	0.731766	0.031303	0.001128
6738	'TROVE2'	mRNA	0.727425	7.09E-04	8.02E-06
150465	'TTL'	mRNA	0.72455	0.001871	2.83E-05
10314	'LANCL1'	mRNA	0.722174	5.47E-04	5.77E-06
79616	'CCNJL'	mRNA	0.720735	0.033289	0.001231
130576	'LYPD6B'	mRNA	0.719755	0.029233	0.00101
5834	'PYGB'	mRNA	0.719112	0.00861	1.98E-04
54813	'KLHL28'	mRNA	0.718109	0.036911	0.001445
5861	'RAB1A'	mRNA	0.717436	4.70E-05	2.75E-07
339324	'ZNF260'	mRNA	0.716764	0.0064	1.38E-04
84725	'PLEKHA8'	mRNA	0.716162	6.43E-04	7.08E-06
51776	'MAP3K20'	mRNA	0.716123	0.021088	6.65E-04
404093	'CUEDC1'	mRNA	0.715381	0.03388	0.001268
55604	'CARMIL1'	mRNA	0.713548	0.033481	0.001247
390	'RND3'	mRNA	0.713114	0.003172	5.44E-05
8050	'PDHX'	mRNA	0.711182	0.00446	8.36E-05
126731	'CCSAP'	mRNA	0.708017	0.005695	1.16E-04

3177	'SLC29A2'	mRNA	0.705018	0.013221	3.48E-04
23526	'ARHGAP45'	mRNA	0.702883	0.047306	0.002044
260334	'TUBB8P12'	mRNA	0.700498	0.033384	0.00124
9061	'PAPSS1'	mRNA	0.696412	0.017454	5.19E-04
26509	'MYOF'	mRNA	0.695457	0.021001	6.60E-04
8870	'IER3'	mRNA	0.689608	0.020399	6.37E-04
2934	'GSN'	mRNA	0.688056	0.019839	6.17E-04
29995	'LMCD1'	mRNA	0.687143	0.033201	0.001224
255520	'ELMOD2'	mRNA	0.682708	6.05E-04	6.60E-06
23398	'PPWD1'	mRNA	0.682542	0.005196	1.01E-04
148534	'TMEM56'	mRNA	0.680127	0.012419	3.17E-04
3399	'ID3'	mRNA	0.679944	0.012548	3.21E-04
6341	'SCO1'	mRNA	0.677923	0.046405	0.001989
123606	'NIPA1'	mRNA	0.676438	0.035129	0.001342
55544	'RBM38'	mRNA	0.674168	0.041247	0.00167
8503	'PIK3R3'	mRNA	0.672985	0.049076	0.002153
91010	'FMNL3'	mRNA	0.672491	0.020996	6.59E-04
55827	'DCAF6'	mRNA	0.672006	0.008696	2.02E-04
56938	'ARNTL2'	mRNA	0.670559	0.037359	0.001474
6659	'SOX4'	mRNA	0.669991	4.07E-04	4.03E-06
54994	'GID8'	mRNA	0.669919	0.002324	3.75E-05
80325	'ABTB1'	mRNA	0.669431	0.03648	0.00142
10198	'MPHOSPH9'	mRNA	0.667921	0.013661	3.67E-04
65078	'RTN4R'	mRNA	0.666573	0.042216	0.00173
403313	'PLPP6'	mRNA	0.665738	0.033658	0.001258
11183	'MAP4K5'	mRNA	0.664364	0.007972	1.80E-04
1846	'DUSP4'	mRNA	0.66295	0.029541	0.001026
1388	'ATF6B'	mRNA	0.659257	0.013713	3.70E-04
10129	'FRY'	mRNA	0.65896	0.019499	5.93E-04
2200	'FBN1'	mRNA	0.65749	0.014465	3.93E-04
54819	'ZCCHC10'	mRNA	0.656911	0.03648	0.001422
23080	'AVL9'	mRNA	0.654645	0.008411	1.92E-04
7106	'TSPAN4'	mRNA	0.654021	0.042041	0.00172
403341	'ZBTB34'	mRNA	0.650467	0.013713	3.70E-04
607	'BCL9'	mRNA	0.649688	1.31E-04	9.94E-07
5045	'FURIN'	mRNA	0.644115	0.044274	0.001851
79776	'ZFHX4'	mRNA	0.64235	0.022778	7.39E-04
10098	'TSPAN5'	mRNA	0.64145	0.036911	0.001445
7431	'VIM'	mRNA	0.640675	0.006202	1.31E-04
10380	'BPNT1'	mRNA	0.639658	0.001404	1.98E-05
10336	'PCGF3'	mRNA	0.636288	9.42E-04	1.16E-05
4162	'MCAM'	mRNA	0.631716	0.042216	0.001736
50862	'RNF141'	mRNA	0.627821	0.03089	0.001104
374986	'MIGA1'	mRNA	0.62651	0.032287	0.001171
9351	'SLC9A3R2'	mRNA	0.626087	0.042821	0.00177
55103	'RALGPS2'	mRNA	0.625707	0.011487	2.86E-04

151648	'SGO1'	mRNA	0.611784	0.015578	4.46E-04
8575	'PRKRA'	mRNA	0.611292	0.026561	8.96E-04
57221	'ARFGEF3'	mRNA	0.608648	0.007403	1.66E-04
3075	'CFH'	mRNA	0.605865	0.031168	0.001121
10038	'PARP2'	mRNA	0.602564	0.00302	5.11E-05
5939	'RBMS2'	mRNA	0.601483	0.011805	2.97E-04
9074	'CLDN6'	mRNA	0.601381	0.036295	0.001408
51719	'CAB39'	mRNA	0.60052	0.03089	0.001101
3964	'LGALS8'	mRNA	0.600311	0.006028	1.26E-04
56892	'TCIM'	mRNA	0.599466	0.029857	0.001046
6002	'RGS12'	mRNA	0.592869	0.024556	8.13E-04
254427	'PROSER2'	mRNA	0.592119	0.012887	3.35E-04
7525	'YES1'	mRNA	0.591389	0.010712	2.64E-04
1000	'CDH2'	mRNA	0.588128	9.73E-04	1.21E-05
55798	'METTL2B'	mRNA	0.588062	0.019839	6.17E-04
7846	'TUBA1A'	mRNA	0.582168	0.019568	6.01E-04
57552	'NCEH1'	mRNA	0.578914	0.01843	5.53E-04
3842	'TNPO1'	mRNA	0.57798	0.007171	1.60E-04
214	'ALCAM'	mRNA	0.572134	0.035479	0.001362
57798	'GATAD1'	mRNA	0.571916	0.027444	9.37E-04
143686	'SESN3'	mRNA	0.570882	0.015903	4.57E-04
2589	'GALNT1'	mRNA	0.570723	0.008099	1.83E-04
143662	'MUC15'	mRNA	0.570209	0.015039	4.22E-04
23384	'SPECC1L'	mRNA	0.570052	0.01019	2.47E-04
375790	'AGRN'	mRNA	0.569201	0.008429	1.94E-04
5774	'PTPN3'	mRNA	0.564426	0.016874	4.89E-04
283464	'GXYLT1'	mRNA	0.561687	0.025281	8.44E-04
7071	'KLF10'	mRNA	0.561642	0.036122	0.001397
115353	'LRRC42'	mRNA	0.561436	0.014533	3.97E-04
23233	'EXOC6B'	mRNA	0.557338	0.019833	6.14E-04
3801	'KIFC3'	mRNA	0.557332	0.042041	0.001719
1265	'CNN2'	mRNA	0.556493	0.002252	3.61E-05
23199	'GSE1'	mRNA	0.551903	0.019184	5.77E-04
54532	'USP53'	mRNA	0.549159	0.046018	0.001957
11145	'PLA2G16'	mRNA	0.547645	0.047886	0.002081
84733	'CBX2'	mRNA	0.541266	0.024556	8.13E-04
23235	'SIK2'	mRNA	0.53628	0.010196	2.48E-04
54708	'MARCH5'	mRNA	0.535516	0.016891	4.93E-04
170960	'ZNF721'	mRNA	0.535238	0.037013	0.001453
10159	'ATP6AP2'	mRNA	0.534354	0.013593	3.64E-04
9732	'DOCK4'	mRNA	0.530081	0.026068	8.75E-04
22931	'RAB18'	mRNA	0.528929	0.029769	0.001036
55765	'INAVA'	mRNA	0.528023	0.025469	8.51E-04
221833	'SP8'	mRNA	0.527676	0.029857	0.00105
3996	'LLGL1'	mRNA	0.526652	0.036379	0.001413
727	'C5'	mRNA	0.519015	0.012238	3.11E-04

56172	'ANKH'	mRNA	0.518793	0.017388	5.08E-04
11044	'TENT4A'	mRNA	0.513366	0.043547	0.001805
22859	'ADGRL1'	mRNA	0.512756	0.029857	0.00105
9693	'RAPGEF2'	mRNA	0.511821	0.00401	7.35E-05
2000	'ELF4'	mRNA	0.511083	0.017454	5.18E-04
10552	'ARPC1A'	mRNA	0.505409	0.023718	7.76E-04
84925	'DIRC2'	mRNA	0.499492	0.032424	0.001181
128637	'TBC1D20'	mRNA	0.489174	0.046592	0.002
8842	'PROM1'	mRNA	0.480332	0.015477	4.42E-04
25814	'ATXN10'	mRNA	0.472979	0.048045	0.002093
1964	'EIF1AX'	mRNA	0.469695	0.041355	0.001682
23161	'SNX13'	mRNA	0.461928	0.04484	0.001893
10413	'YAP1'	mRNA	0.449236	0.024556	8.14E-04

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Review

Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis

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Abstract: NF-E2-related factor 2 (NRF2) is a basic leucine zipper transcription factor, a master regulator of redox homeostasis regulating a variety of genes for antioxidant and detoxification enzymes. NRF2 was, therefore, initially thought to protect the liver from oxidative stress. Recent studies, however, have revealed that mutations in NRF2 cause aberrant accumulation of NRF2 in the nucleus and exert the upregulation of NRF2 target genes. Moreover, among all molecular changes in hepatocellular carcinoma (HCC), NRF2 activation has been revealed as a more prominent pathway contributing to the progression of precancerous lesions to malignancy. Nevertheless, how its activation leads to poor prognosis in HCC patients remains unclear. In this review, we provide an overview of how aberrant activation of NRF2 triggers HCC development. We also summarize the emerging roles of other NRF family members in liver cancer development.

Keywords: NF-E2-related factor 2; transcription factor; redox homeostasis; oxidative stress; hepatocellular carcinoma

1. Introduction

Liver cancer is one of the most troublesome human malignancies, with an annual incidence of around 600,000 worldwide (https://doi.org/10.3322/canjclin.55.2.74). Among different types of liver cancer, hepatocellular carcinoma (HCC) is the sixth most common malignancy of liver (http://gco.iarc.fr/) and the eighth leading cause of cancer-related deaths in Europe (http://gco.iarc.fr/today). Chronic hepatitis B (HBV) or C (HCV) virus infection, alcohol intake, diabetes, fatty liver disease,

and chronic liver injury cause permanent hepatocellular damage, hepatocyte regeneration, and inflammation, which are key risk factors for HCC. Infection with HBV or HCV and alcohol-induced hepatocarcinogenesis are associated with oxidative stress in the liver [1–5]. Oxidative stress also contributes to genomic instability, and the altered gene expression leads to HCC development [6–8]. Moreover, oxidative stress has been suggested to cause cancer-specific gene mutations in the cell cycle, apoptosis, and various processes of the regeneration cycle, which may lead to liver damage [9–12].

NF-E2-related factor 2 (NRF2) transcription factor is activated by oxidative stress, and recent studies have suggested that the aberrant activation of NRF2 triggers hepatomegaly and HCC development [13,14], however, this factor also acts to protect the liver from oxidative stress. The protein interaction of NRF2 and Kelch-like ECH-associated protein 1 (KEAP1) is known to orchestrate the NRF2-dependent oxidative stress response to maintain liver homeostasis. In the presence of oxidative stress, KEAP1 is degraded in the cytoplasm, and due to the degradation, NRF2 is released from KEAP1 [15,16]. Thereafter, NRF2 is phosphorylated and translocates into the nucleus, and induces a series of cytoprotective genes by binding to the antioxidant response element (ARE) after heterodimerization with small musculoaponeurotic fibrosarcoma oncogene homolog (Maf) proteins [17–19]. More importantly, several studies have demonstrated that somatic mutations occur in the coding region of NRF2 and are associated with poor prognoses and overall low survival rates in several cancers [20–23]. The mutations are mostly located in the DLG or ETGE motifs of NRF2 and have been reported to impair the NRF2 binding ability to KEAP1, which in turn leads to aberrant nuclear accumulation of NRF2 [21,23,24]. In this review, we describe how the aberrant transcriptional activation of NRF2 caused by its nuclear accumulation may develop HCC at molecular level.

2. Oxidative Stress-Dependent HCC Pathogenesis

Multiple genetic and epigenetic changes are involved in the development of HCC. Many studies have revealed that oxidative stress is one of the causes linked to tumor initiation and progression by disrupting the normal cellular redox homeostasis [25–27]. Oxidative stress also induces mitochondrial dysfunction, accelerates telomere shortening, causes DNA damage, and is associated with mutations of apoptosis-specific genes in HCC [28–32]. An increased level of reactive oxygen species (ROS), generated by Kupffer cells during hepatic inflammation, has been associated with the progression of liver pathologies [33]. Similarly, oxidative stress disrupts intracellular signaling pathways and contributes to HCC. Alterations in nuclear factor-kappa beta (NF-κB), peroxisome proliferator-activated receptor alpha (PPARα), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK), and transforming growth factor β1 (TGF-β1) pathways due to oxidative stress are commonly associated with HCC, as all these pathways are involved in the activation of cellular proliferation and survival [34–39]. Moreover, HCV infection enhances NF-κB and TGF-β1 expression through the production of ROS and activation of p38-MAPK, ERK, and JNK, and thus promotes the development of hepatic fibrosis [35,37]. Rodent models of DEN/CCl4-induced hepatocarcinogenesis display a significant upregulation of liver-specific NF-κB and TGF-β1/Smad3 signaling [40]. In contrast, HepG2 HCC cells exposed to cadmium telluride quantum dots-induced oxidative stress exhibit decreased levels of Glutathione-SH and Bcl2 and increased expression of NRF2 together with apoptosis induction due to the activation of the MAPK-JNK pathway [36]. These studies suggest that multiple intracellular signaling pathways are activated by oxidative stress, and notably, either carcinogenic or anticarcinogenic pathways are triggered in a context-dependent manner.

3. Dysregulation of NRF2-KEAP1 Physical Interaction Triggers Several Types of Cancer

Several studies have indicated that the NRF2-KEAP1 signaling pathway functions as an oxidative stress sensor [16]. KEAP1 is an adapter protein for the E3 ubiquitin ligase complex that controls the stability and accumulation of NRF2 [41]. Under normal conditions, KEAP1 binds to NRF2 and directs it to Cullin 3-RING E3 (CUL3 E3) ligase for ubiquitination and subsequent proteasomal degradation [42,43]. Upon exposure to oxidative or electrophilic stresses, KEAP1-mediated proteasomal

degradation of NRF2 is inhibited and leads to NRF2-mediated transcription of various genes in several types of tissue, including liver. NRF2 is a member of the Cap'n'Collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors that regulate a wide variety of genes for antioxidant and detoxification enzymes [44,45]. This activity relies on its transactivation capacity and its heterodimeric partner, small MAF transcription factor [46]. The NRF2 protein consists of seven conserved NRF2-ECH homology (Neh) domains. Neh1 contains CNC-bZIP domain via which NRF2 dimerizes with Maf [46]. The consensus binding site of NRF2 is the ARE sequence (TGACNNNGC) [47–49]. The N-terminal region contains the highly conserved Neh2 domain, which negatively regulates the transcriptional activity of NRF2. The Neh2 domain of NRF2 contains DLG and ETGE motifs, which are the binding sites for KEAP1 [50–52]. Alternatively, repression of Nrf2 is achieved by interactions of Neh7 with the DNA-binding domain of retinoic X receptor α [53]. Neh6 is target for E3 ubiquitin ligase β-TrCP leading to degradation [54]. The Neh3–5 domains are thought to bind to transcriptional system [55,56]. KEAP1 is a member of BTB-Kelch family of proteins. The BTB domain is N-terminally situated and is responsible for homodimerization of KEAP1 and interaction with CUL3 [57]. The C-terminal Kelch domain binds to the ETGE motif or DLG motif of NRF2 [51]. The Kelch domain forms a six-bladed β-propeller structure, and this domain is evolutionarily conserved among species. Moreover, among the six Kelch blades, four β-strands are conserved in each blade [58]. Recent studies have provided insight into how mutations disturb the structure of the BTB-Kelch domain that is responsible for NRF2 binding. It has been reported that one single-point mutation, a proline substitution for serine 383 (S383P) in KEAP1, significantly reduces the ability of the Kelch domain to bind with the Neh2 domain of NRF2 [58]. Moreover, KEAP1 substitution mutations of cysteine residues Cys273S/A and Cys288S/A do not affect the ability of KEAP1 to interact with NRF2, but they impair the KEAP1-mediated degradation of NRF2 [59,60]. Notably, many studies have revealed that the loss of interaction between NRF2 and KEAP1 causes tumor development in multiple cancer types. Furthermore, it has been reported that the deletion of Exon2 in NRF2, which reduces interaction with KEAP1, causes tumor development in lung and liver cancer [61]. Together, a tightly regulated balance of NRF2 and KEAP1 interaction is essential to protect cells or tissues from oxidative stress, and the failure of that mechanism (e.g., mutations of critical amino acids) triggers cancer development.

4. Mutations in NRF2 and KEAP1 Functional Domains Induce HCC Development

The International Cancer Genome Consortium (ICGC; https://icgc.org/) has identified somatic mutations in the NRF2 gene of human lung, liver, breast, head, and neck cancer patients. Notably, these mutations were mostly located within the DLG and ETGE motifs, which provides NRF2 with gain-of-function activity in different cancer types [21,23,62]. Therefore, we surveyed the ICGC database and compiled the HCC somatic mutations found in DLG and ETGE motifs (Table 1). KEAP1 mutations are also found within the BTB, IVR, and Kelch domains (Figure 1). Comprehensive genomic analyses have identified somatic mutations in the *NRF2* and *KEAP1* genes in various types of cancer [21,23,63]. Interestingly, mutations in *KEAP1* and *NRF2* are mutually exclusive and rarely occurred in the same cancer cell [64]. Whole-exome sequencing has identified 6.4% of the somatic mutations in *NRF2* in HCC patients [24]. Somatic *NRF2* and *KEAP1* mutations were most often found in lung squamous cell carcinoma, esophageal carcinoma, uterine corpus endometrial carcinoma, lung adenocarcinoma, head and neck cancers, and HCC; the overlapping somatic *NRF2* or *KEAP1* mutations are associated with a sustained NRF2 activation phenotype [21,23]. In 995 lung cancer cases, 423 cases were estimated to have constitutive NRF2 activation, and 165 cases harbored either *NRF2* or *KEAP1* mutations with higher expression of NRF2 target genes, including Aldo-Keto Reductase Family 1 Member B10 (*AKR1B10*), Aldo-Keto Reductase Family 1 Member B15 (*AKR1B15*), Glutathione Peroxidase 2 (*GPX2*), Thioredoxin Reductase 1 (*TXNRD1*), Glutamate-Cysteine Ligase Modifier Subunit (*GCLM*), and Glutamate-Cysteine Ligase Catalytic Subunit Glutamate-Cysteine Ligase Catalytic Subunit (*GCLC*) [21]. It has been reported that NRF2 gain-of-function mutations are one of the possible triggers of HCC [65,66]. In an experimental rat model of hepatocarcinogenesis, it was found that the *NRF2* gene was frequently

mutated or activated during the early stage of the tumorigenic process [66]. This suggests that NRF2 plays a potent role for the initiation of HCC and is mandatory for the development of preneoplastic lesions. The role of constitutive NRF2 activation is well established in chemo- and radio resistance in various tumors [67,68]. In Lung Squamous Cell Carcinoma (LSCC) mice models developed by *KEAP1* deletion, the persistent activation of NRF2 has been suggested to contribute to increased tumor formation, metastasis, and resistance to oxidative stress and irradiation [67]. The NRF2 DLG and ETGE motifs have been reported as driver mutations in several types of cancer including HCC [62,65,66]. A recent CRISPR/Cas9 genome-wide screening study demonstrated that KEAP1 depletion causes aberrant NRF2 transcriptional activity as well as high chemoresistance. Upregulation in NRF2-target gene expression including NAD(P)H-quinone oxidoreductase 1 (*NQO1*), *GPX2*, and *TXNRD1* was also observed [69]. Moreover, microdeletion of *NRF2* exon 2 (where the DLG and ETGE domains are located) in JHH cells is associated with increased NRF2 target gene expression and is similar to HCC cell lines containing *KEAP1* mutations [61]. NRF2 DLG and ETGE mutations that demonstrate a loss of interaction with KEAP1, are localized to the nucleus and exert sustained target gene activation. In fact, *NRF2* mutations occur frequently in the DLG or ETGE motifs and activate Nqo1, Gclc, and Gsta4 pathways. This suggests that *NRF2* mutations are able to enhance NRF2 transcriptional activity [62]. Mutation in the DLG motif of NRF2 induces ARE-regulated PPP enzyme transcription, which is required for cell growth and proliferation [65,70]. NRF2 also translocates into the nucleus in HCV-infected hepatocytes and causes mouse double minute 2 homolog (MDM2)-mediated retinoblastoma protein (Rb) degradation. This subsequently induces HCC progression [71]. Overall, these findings suggest that either the *NRF2* DLG/ETGE mutations or KEAP1 mutations induce aberrant NRF2 activity and may induce HCC through NRF2-ARE pathway activation. Moreover, methylation of the *KEAP1* promoter has been reported to induce cancer development and chemo- and radio- resistance in multiple cancer types [72,73]. It has been determined that the most frequent somatic mutations found in HCC are telomerase reverse transcriptase (*TERT*) promoter mutations, identified in premalignant lesions in cirrhosis [74]. TERT is essential for telomere elongation and maintenance during cell division. *TERT* promoter mutations are associated with increased telomerase activity, which displays enhanced *NRF2* expression and inhibited glycogen accumulation [75]. As such, it appears there are many possible pathways to trigger liver cancer via aberrant NRF2 transcriptional activity, and further phenotypic validation of the roles of these pathways in liver cancer development merits investigation.

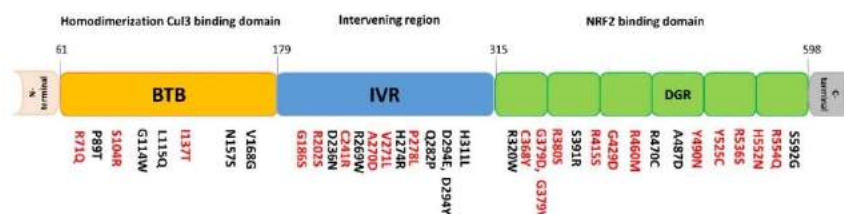

Figure 1. Human Kelch-like ECH-associated protein 1 (KEAP1) mutations (liver cancer) identified by the International Cancer Genome Consortium. The amino acid positions of the identified mutations of KEAP1 are shown and the amino acid positions in red color indicate the location of mutations that are conserved among several species (Human, Mouse, Bovine, and Zebrafish). The ^{61}BTB^{179} domain is required for homodimerization of Keap1 by interactions with the Cul3 based E3 ubiquitin ligase system. The ^{315}DGR^{598} or 6 Kelch-repeat domain binds to NRF2 through Neh2 domain of NRF2. ^{180}IVR^{314} domain between BTB and DGR domain important sensing oxidative stress and xenobiotic stimuli via modification of its four cysteine residues by electrophiles.

Table 1. NF-E2-related factor 2 (NRF2) mutations found in NRF2-ECH homology 2 (Neh2) domain ETGE and DLG motif of hepatocellular carcinoma (HCC) patients (International Cancer Genome Consortium (ICGC) database).

Mutation ID	Genomic DNA Change	Type	Motif	Substitution to	Clinical Significance	Occurrence of Mutation
MU871836	chr2:g.178098959T > C	single base substitution	DLG	G	Likely pathogenic	5
MU866686	chr2:g.178098953C > T	single base substitution	DLG	E	ND	2
MU1327674	chr2:g.178098960C > T	single base substitution	DLG	N	Likely pathogenic	2
MU29615597	chr2:g.178098959T > G	single base substitution	DLG	A	ND	1
MU83818151	chr2:g.178098954C > T	single base substitution	DLG	R	Pathogenic/Likely pathogenic	1
MU1324215	chr2:g.178098960C > G	single base substitution	DLG	H	Likely pathogenic	1
MU830878	chr2:g.178098956A > C	single base substitution	DLG	R	ND	3
MU1330977	chr2:g.178098957G > A	single base substitution	DLG	F	ND	1
MU825005	chr2:g.178098800T > C	single base substitution	ETGE	G	ND	4
MU7421282	chr2:g.178098809T > C	single base substitution	ETGE	G	ND	2
MU29777568	chr2:g.178098806G > A	single base substitution	ETGE	I	ND	2
MU29708787	chr2:g.178098799T > G	single base substitution	ETGE	D	ND	2
MU1890585	chr2:g.178098804C > A	single base substitution	ETGE	C	ND	2
MU1332094	chr2:g.178098804C > T	single base substitution	ETGE	S	ND	2
MU3162037	chr2:g.178098809T > A	single base substitution	ETGE	V	Likely pathogenic	2
MU128988244	chr2:g.178098803C > G	single base substitution	ETGE	A	ND	1
MU1804262	chr2:g.178098799T > A	single base substitution	ETGE	D	ND	1
MU41238347	chr2:g.178098804C > G	single base substitution	ETGE	R	ND	1
MU1817004	chr2:g.178098807T > G	single base substitution	ETGE	P	ND	1
MU112734927	chr2:g.178098809T > G	single base substitution	ETGE	A	ND	1
MU871364	chr2:g.178098803C > A	single base substitution	ETGE	V	ND	1
MU2689228	chr2:g.178098800T > G	single base substitution	ETGE	A	ND	1

5. Aberrantly Activated NRF2 Targets Several Gene Expressions in HCC

As summarized in Table 2, it has been suggested that excessive NRF2 transcriptional activity promotes the development of liver cancer by regulating the expression of various genes. During oxidative stress, cells initiate an adaptive response that upregulates expression of a large array of cytoprotective genes. The battery of genes is regulated through NRF2 binding to the ARE consensus binding sequence, which includes glutamate-cysteine ligase (*GCL*), thioredoxin reductase 1, NAD(P)H-quinone oxidoreductase 1 (*NQO1*), and heme oxygenase-1 (*HO-1*) [76]. In healthy cells, HO-1 exhibits its cytoprotective effect through detoxification and ROS scavenging mechanisms that decrease the possibility of tumor initiation. However, in a tumor microenvironment, sustained NRF2 expression persists due to stress, which tightly regulates the expression of HO-1. Thus, in growing

tumors, HO-1 plays a pro-tumorigenic role by increasing tumor cell proliferation and metastasis and blocking cell death [77,78].

The extracellular matrix metalloproteinase MMP-9 demonstrates a crucial role in HCC invasion and progression [79]. Many studies have suggested that MMP-9 is a prognostic biomarker to predict tumor invasiveness and recurrence in HCC patients [80,81]. Moreover, it was found that 98 samples displayed MMP-9 positive expression in 143 HCC tissue samples. It has been reported that the *MMP-9* gene expression has a strong correlation with the metastatic potential of HCC cell lines (MHCC97-L, MHCC97-H, and HCCLM6) and its expression was significantly increased in an in vitro HCC invasion model [79]. This evidence suggests that MMP-9 plays a critical role in HCC invasiveness and metastasis. A positive correlation between *NRF2* and *MMP-9* expression in human HCC samples and HCC cell lines has been reported [82]. Moreover, NRF2-mediated induction of MMP-9 plays an important role in cell proliferation and invasion [82].

The presence of a strong correlation between NRF2 and the PI3K-Akt signaling pathway has been demonstrated in driving metabolic gene expression and increased cellular proliferation [83]. In many cancers, the PI3K-Akt pathway is constitutively active and may be responsible for increasing the nuclear levels of NRF2 by inhibiting the GSK3-mediated degradation of NRF2.

Peroxiredoxin 1 (*PRDX1*), a NRF2 target gene, is known to act as an oxidative stress sensor and promote liver cancer growth [84,85]. In HepG2 cells, a significant decrease in cell proliferation and upregulation of proapoptotic genes BAX and Caspase 3 were observed when *PRDX1* was silenced, suggesting the prosurvival and tumorigenic roles of *PRDX1*. Furthermore, proteomic analysis has revealed changes in expression and oxidation of proteins involved in central metabolism and tumor growth, indicating that PRDX1 is one of metabolic reprogramming factors in cancer cells [86].

Methylenetetrahydrofolate dehydrogenase 1–like (MTHFD1L) is an enzyme, involved in the folate cycle which is also known as a target gene of NRF2. Transcriptome sequencing of HCC patients and The Cancer Genome Atlas (TCGA) data showed that MTHFD1L is significantly overexpressed in different cancers, including HCC. During HCC cell proliferation, the folate cycle provides nutrition to the cells by supplying metabolites for NADPH and DNA synthesis. The *MTHFD1L* promoter has three ARE sequence elements and is transcriptionally controlled by NRF2. Genetic knockdown (KD) of either *NRF2* or *MTHFD1L* or inhibition of the folate cycle through antifolate drug can inhibit liver cancer cell proliferation by increasing oxidative stress, altering the metabolic program, and sensitizing HCC cells to sorafenib treatment [87].

In cancer cells, the aberrant activation of NRF2 helps their metabolic adaptations through regulating the key genes involved in glucose metabolism pathways. It has been reported that the effect of NRF2 on the regulation of glucose metabolism is partly through suppressing the transcription of miR-1 and miR-206. The miR-1 and miR-206 regulate their target genes of the PPP (pentose phosphate pathway) (Glucose-6-phosphate dehydrogenase *(G6PD)*, Phosphogluconate Dehydrogenase *(PGD)*, Transketolase *(TKT)*, and Transaldolase 1 *(TALDO1)*) [88], suggesting that suppression of these miRNAs expression caused by NRF2 aberrant activation may enhance tumor development through glucose metabolism deficiency. Additionally, suppressed expression of miR-1 also has been proposed to be important in liver cancer cell growth [89]. On the other hand, in a resistant-hepatocyte rat model of HCC, microRNA profiling revealed an upregulation of miR-200a at the very early stage of tumorigenesis. miR-200a downregulates KEAP1 in several cancer types including HCC [90,91] and it promotes induced expression of *NQO*-1 and *GSTA*-4, and *GCLC* genes which are the direct target of NRF2 [91]. These findings suggest that NRF2 plays important roles in the promotion of liver cancer cell growth through the regulation of several genes and microRNAs.

Table 2. List of NRF2 target genes and their effects on HCC development.

NRF2 Target Genes	Effect of Gene Expression	ARE in Promoter
NAD(P)H dehydrogenase, quinone 1 (*NQO1*) and Hemeoxigenase 1 (*HO-1*) [92]	Constitutive activation of NRF2 by hepatotoxin contributes to the upregulation of *NQO1* and *HO-1*. This promotes liver cancer cell growth.	YES
B-cell lymphoma-extra-large (*Bcl-xL*) [68]	Antioxidant stabilized NRF2 increases the expression of *Bcl-xL* gene which causes reduction in apoptosis, increase cell survival, and drug resistance in Hepa1-6 cells.	YES
Glutathione S-transferase A4 (*GSTA4*) [93]. Glutamate-cysteine ligase (*GCLC*) [91], [94]	Constitutive activation of NRF2 in preneoplastic lesions of HCC increases the expression of *GSTA4* and *GCLC* and promotes HCC cell growth.	YES
Placental glutathione S-transferase (*GST-P*) [95,96]	NRF2/MafK heterodimer activates *GST-P* gene (a prominent tumor marker for hepatocarcinogenesis) through the binding with GPE1 enhancer during hepatocarcinogenesis.	YES
Matrix metalloproteinases-9 (*MMP-9*) [82], [97]	Up-regulation of NRF2 in HepG2 cells increases the *MMP-9* expression which promotes the cell invasion ability of HCC. In response to ROS, NRF2 induces *MMP-9* expression in HepG2 cells, which contributes to cancer cell migration and invasiveness.	YES
Platelet-Derived Growth Factor-A (*PDGFA*) [98]	NRF2 promotes *PDGFA* transcription by interacting with SP1 thus promotes HCC proliferation.	NO (Through interaction with Sp1)
Prostaglandin reductase-1 (*PTGR1*) [99]	*PTGR1* expression regulated by NRF2 regulates antioxidant responses to promote cell proliferation in HCC. PTGR1 overexpression in HCC increases cell proliferation and develop resistance to ROS-induced cell death.	YES
Peroxisome proliferator-activated receptor γ (*PPARγ*) [100]	Overexpression of NRF2 in HFD increases the expression of *PPARγ* and accumulates hepatic triglyceride which initiates NAFLD.	NO (Correlation was tested)
26S proteasome non-ATPase regulatory subunit 10 (*PSMD10*) or Gankyrin [101]	Increased NRF2 activity up-regulates gankyrin expression in HCC.	YES
Mouse double minute 2 homolog (*MDM2*) [71,102]	During HCV infection, NRF2 translocates into the nucleus and induces MDM2-mediated retinoblastoma protein (Rb) degradation. This induces HCC progression.	YES

6. Aberrant Activation of NRF2, a Critical Regulator of Lipid and Cholesterol Metabolism, Leads to HCC

Non-alcoholic fatty liver disease (NAFLD) is considered one of the risk factors of HCC. NAFLD includes a variety of liver pathologies including the accumulation of triglycerides in the hepatocytes, liver inflammation, and non-alcoholic steatohepatitis (NASH) that leads to cirrhosis and thereafter HCC [103–105]. The most important mechanism of NASH pathogenesis is increased hepatic iron accumulation, as well as oxidative DNA damage [106]. Highly proliferative cancer cells show strong affinity towards lipid and cholesterol metabolisms [107], and high levels of cholesterols and lipids are now considered hallmarks of many aggressive cancers [108–112]. It is also evident that hepatic lipid and fatty acid overload are related to the development of HCC [113]. The pathogenic role of NRF2 for the initiation and development of hepatic steatosis was described previously [114]. Additionally, enhanced NRF2 activity augments hepatic steatosis and increased lipid deposition in the liver has been reported. In leptin-deficient mice, constitutive activation of NRF2 via KEAP1-KD established insulin resistance, inhibited the accumulation of lipids in adipose tissue, and subsequently increased hepatic steatosis. [114]. Moreover, dysfunction in the starvation-induced hepatic lipid droplets (LDs) synthesis in liver-specific Atg5 (L-Atg5)-deficient mouse livers was associated with sustained NRF2 activation [115]. Though it has been reported that NRF2 activation may reduce cholesterol injury by regulating the lipid homeostasis, how the hyperactivation of this transcription factor in NAFLD leads to HCC needs to be further clarified.

NAFLD results from unbalanced lipid metabolism. Forkhead box protein A1 (FOXA1) is a triglyceride synthesis inhibitor, and it is well known to lower fatty acid uptake [116]. Thus, FOXA1 is thought to be an antisteatotic regulator in lipid metabolic pathways hepatocytes. Notably, it was found that excessive cholesterol synthesis causes the accumulation of NRF2. The accumulated NRF2 suppresses the expression of FOXA1 [117], and the downregulation of FOXA1 has been found in human and rat NAFLD [116]. In this regard, the disruption of lipid metabolism and oxidative stress have been reported as the main causes of NAFLD, and NRF2 is related to lipid homeostasis [118,119]. Peroxisome proliferator-activated receptor gamma (PPARγ) is also one of the most studied lipid metabolism regulators in hepatocytes and it contributes to the development of NAFLD [120]. Interestingly, it has been reported that *PPARγ* gene expression is regulated by NRF2 [121]. Moreover, it was found that liver-specific *Nrf2*-KO mice with high-fat diet (HFD) had less steatosis and inflammation with less hepatic triglyceride levels and decreased PPARγ activity [100]. Furthermore, constitutively activated NRF2 signaling in *Keap1*-KD mice fed a HFD exhibited greater lipogenic gene expression, inflammation, and increased hepatic steatosis [122]. These findings suggest that aberrant activation of NRF2 helps to trigger development of NASH or NAFLD, therefore, gain-of-function type mutations in NRF2 may initiate the development of HCC by inducing NASH or NAFLD. Nevertheless, since the NRF2-controlled gene network contributing to the promotion of HCC is not clear, further studies are needed to investigate how NRF2 induces NASH or NAFLD at the molecular level.

7. Emerging Mechanism of NRF2 Activation-Induced HCC

ROS is usually considered to be carcinogenic and several chemopreventive strategies for the usage of NRF2 have been proposed [123,124]. Since NRF2 is widely known to be a potent protector in anti-oxidative response, a question arises as to how NRF2 DLG and ETGE mutations lead to increased malignancy of HCC and trigger its resistance to chemotherapy. This issue has been discussed very intensively in several reviews [14,125] and an excellent hypothesis is proposed by Sporn and his group that the role of NRF2 can be altered depending on the stage of tumor progression [126]. They proposed a model in which enhancement of NRF2 activity can protect advanced tumors from the cytotoxic effects of ROS that are induced by oncogenic signaling whereas NRF2 activation acts as protective for tumor establishment in normal condition. Interestingly, oncogenic gene mutations such as K-RasG12D, B-RafV619E enhanced transcription of NRF2 with elevated NRF2 target gene expression and lowered intracellular ROS [127]. Therefore, it is assumed that aberrant transcriptional activity induced by high expression or mutation of NRF2 may lead to malignancy in combination with other factors (for example, mutation of oncogene leads cells to early cancer state or abnormality of metabolic state changes in cellular environment). In these conditions, the cells can be shifted to malignancy when they are induced to become HCC progenitor cells. Interestingly, it has been shown that elevated p62 levels aberrantly activate NRF2 transcriptional activity, which induces HCC pathogenesis by accelerating the survival of HCC-initiating cells [128]. p62, which is encoded by Sequestosome-1 (SQSTM1), is an autophagy adaptor. It activates NRF2 through inactivation of Keap1 [129]. Autophagy is an evolutionary conserved cellular mechanism that maintains cell homeostasis by targeting damaged organelles or mistranslated proteins for lysosomal degradation. Atg7 deletion mice develop hepatocellular adenoma accompanied by aberrant accumulation of p62 followed by NRF2 activation [130]. The study further elucidated the role of p62 in aberrant activation of NRF2 in HCC. The persistent activation of NRF2 is associated with p62 accumulation and the development of HCC in vitro [130]. Furthermore, high levels of p62 expression activates NRF2 and mTORC1 in HCC [128]. Consequently, this NRF2 activation spares HCC-initiating cells from oxidative stress-induced cell death [128]. This is supported by the fact that the kinase-dead mutation of p62 (S349A) in Human hepatoma cell line-1 (Huh-1) cells significantly reduces colony formation capacity with decreased *NQO1* mRNA expression [131]. Moreover, a xenograft experiment using a nude mouse demonstrated that the tumor formation capacity of mutant Huh-1 cells (p62 KO and S349A) is reduced as compared to the wild-type. Additionally, p62-mediated NRF2 activation in HCC cells facilitates the glucuronate pathway and glutathione synthesis in HCV

positive HCC [132]. NRF2 activation contributes to metabolic reprogramming in HCC harboring phosphorylated p62 [133]. This leads to increased cell proliferation and increases tolerance to anti-cancer drugs in HCC [132]. These findings clearly demonstrate that sustained activation of NRF2 by p62 activation is responsible for HCC pathology, suggesting that NRF2 and KEAP1 mutations, as well as aberrant p62 activation enhance the growth of HCC cells through metabolic dysregulation. NRF2 DLG and ETGE mutations lose the capacity to interact with KEAP1 and localize mainly to the nucleus. Accordingly, these mutations may activate NRF2 target gene expression and exhibit a similar phenotype to p62 activation in the liver. Future studies on the effect of NRF2 DLG and ETGE mutations in combination with autophagy and/or other cellular function in HCC are warranted.

8. Emerging Roles of the CNC Family of Transcription Factors in HCC

The CNC bZIP family of transcription factors, which comprises four closely related factors, NRF1, NRF2, NRF3, and p45 NF-E2, have developmental and homeostatic functions [134,135]. The CNC gene encodes different proteins, with evolutionary conservation between *Drosophila* CNC isoform and mammalian NRF1, NRF2, and NRF3 [136]. With similar binding and expression profiles, NRF1, NRF2, and NRF3 reside outside of the nucleus under normal conditions [137]. NRF2 resides in the cytoplasm and NRF1 and NRF3 in the endoplasmic reticulum [137,138]. All three transcription factors are essential for maintaining redox homeostasis and directing cellular stress responses. Much like NRF2, NRF1 contains the Neh2 domain and NRF3 does not [139]. NRF-encompassing amino acids 171–244 of Neh2 share 72% homology with the Neh2 domain of NRF2 [137]. In addition, NRF1 has conserved DLG and ETGE motifs within the Neh2 domain, which are essential for KEAP1–NRF1 interaction [140,141]. Moreover, a study indicated that NRF1 expression was significantly reduced in KEAP1-KO H1299 cells, suggesting that KEAP1 stabilizes NRF1 [142]. On the other hand, it has been reported that cytoplasmic localization of NRF1 is independent of KEAP1 whereas KEAP1 physically interacts with NRF1 [137]. Therefore, it is still not clear whether KEAP1 regulates NRF1 function and further study is needed. NRF1 and NRF2 have overlapping targets binding ARE-containing genes, but have distinctive roles [143]. It has also been reported that NRF1 and NRF2 simultaneously control the basal expression of ARE-containing genes in fibroblasts [144]. Likewise, NRF1-3 are known to regulate proteasome gene expression [145–147]. Moreover, the role of NRF1 and NRF3 has been indicated in human cancers, including HCC [148–150]. These results suggest that NRF1 and NRF3 mutations and aberrant gene expressions may trigger HCC. The molecular regulation and biological function of NRF3 in cancer cells have been elucidated [150–152]. When exposed to stress, NRF3 translocates to the nucleus, heterodimerizes with the small Maf proteins similarly to NRF2 via ARE, and activates U2AF homology motif kinase 1 (*UHMK1*) gene expression [150]. This study suggests that NRF3 functions as an inducible transcription factor in cancer progression. Moreover, analysis of TCGA data revealed that NRF3 was highly expressed in HCC tissues, and its expression was positively correlated with tumor grade and stage [149]. In addition, NRF3 deficiency has been revealed to predispose to T-cell lymphoblastic lymphoma when exposed to carcinogens [153]. Likewise, liver-specific inactivation of the *Nrf1* gene in adult mice has been reported to trigger NASH [154], suggesting that the proper activity of NRF1 and NRF3 blocks carcinogenesis, including liver cancer. Therefore, the roles of NRF1 and NRF3 and their mutational effects in HCC merit investigation.

9. Conclusions

In this review, we discussed the current evidence on how aberrant NRF2 transcriptional activity causes HCC development (Figure 2). Moreover, we reviewed the mutations found in ICGC databases in the specific domain that is essential for the KEAP1-NRF2 interaction impact on HCC development. The aberrant activation of NRF2, the dark side of this protein expression, induces the transcription of a series of cytoprotective and xenobiotic-metabolizing genes. Furthermore, align with NRF2, other CNC family members, NRF1 and NRF3, are also dysregulated during HCC development. Hepatocytes are in a continuous struggle to maintain cellular homeostasis, owing to diverse physiological functions of

the liver. In a diseased microenvironment, when NRF2 over-activation is induced through mutation, epigenetic changes, competition or other constitutive changes, and cellular responses are variable. Thus, how the hyperactivity of NRF2 leads to drug resistance and tumor development is the hotspot of future research. Further studies are needed to clarify the underlying mechanisms and investigate the role of NRF2 mutations in the development of liver cancer.

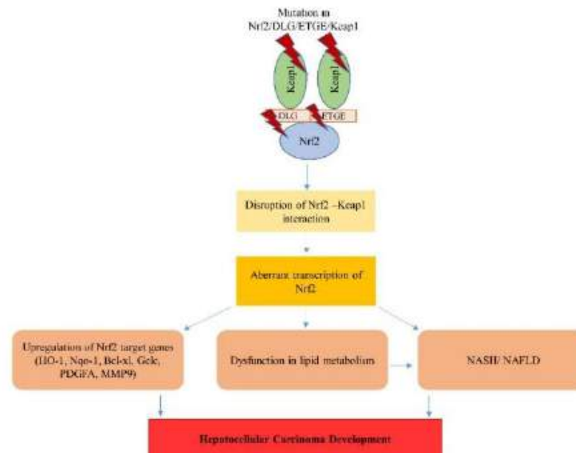

Figure 2. Mutations in NRF2 or Keap1 cause aberrant accumulation of NRF2 in the nucleus that leads to an increase in NRF2 target genes. This aberrant activation of NRF2 dysregulates the lipid metabolism responsible for the non-alcoholic steatohepatitis (NASH)/non-alcoholic fatty liver disease (NAFLD) pathology. Consequently, these events lead to the development of HCC.

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Abbreviations

AKR1B10	Aldo-Keto Reductase Family 1 Member B10
AKR1B15	Aldo-Keto Reductase Family 1 Member B15
ARE	Antioxidant response element
Atg5	Autophagy Related 5
Atg7	Autophagy Related 7
BAX	BCL2 Associated X
Bcl-xL	B-cell lymphoma-extra-large
Bcl2	B-cell lymphoma 2
bZIP	Basic leucine zipper
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9
CUL3 E3	Cullin 3-RING E3
DEN/CCl4	Diethylnitrosamine/Carbon tetrachloride
ERK	Extracellular signal-regulated kinases
FGF19	Fibroblast growth factor 19
FOXA1	Forkhead box protein A1
G6PD	Glucose-6-phosphate dehydrogenase
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GCLM	Glutamate-Cysteine Ligase Modifier Subunit
GPX2	Glutathione Peroxidase 2
GSK3	Glycogen synthase kinase 3
GST-P	Placental glutathione S-transferase
GSTA4	Glutathione S-transferase A4
HBV	Hepatitis B virus

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HFD	High-fat diet
HO-1	Heme oxygenase-1
Huh-1	Human hepatoma cell line-1
ICGC	International Cancer Genome Consortium
JNK	c-Jun N-terminal kinases
KD	Knockdown
KEAP1	Kelch-like ECH-associated protein 1
KO	Knockout
LSCC	Lung Squamous Cell Carcinoma
Maf	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
Mdm2	Mouse double minute 2 homolog
MMP-9	Matrix metalloproteinase
MTHFD1L	Methylenetetrahydrofolate dehydrogenase 1–like
mTORC1	Mammalian target of rapamycin complex 1
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor-kappa beta
NQO1	NAD(P)H-quinone oxidoreductase 1
NRF1	Nuclear respiratory factor 1
NRF2	NF-E2-related factor 2
NRF3	Nuclear factor-like factor 3
PDGFA	Platelet-Derived Growth Factor-A
PGD	Phosphogluconate Dehydrogenase
PI3K-Akt	Protein Kinase B Phosphoinositide-3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor γ
PPP	Pentose phosphate pathway
PRDX1	Peroxiredoxin 1
PSMD10	26S proteasome non-ATPase regulatory subunit 10
PTGR1	Prostaglandin reductase-1
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SQSTM1	Sequestosome-1
Smad3	SMAD family member 3
TALDO1	Transaldolase 1
TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TGF-β1	Transforming growth factor beta 1
TKT	Transketolase
TXNRD1	Thioredoxin Reductase 1
UHMK1	U2AF homology motif kinase 1
β-TrCP	Beta-transducin repeats-containing proteins

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Mgr Effi Haque
Zakład Embriologii Doświadczalnej
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Instytut Genetyki i Biotechnologii Zwierząt PAN

Oświadczenie

Oświadczam, że w publikacjach stanowiących podstawę mojej rozprawy doktorskiej miałam wiodący wkład

1. **HAQUE, E.**; Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413.

Konceptualizacja, metodologia, walidacja, badanie, gromadzenie i przygotowanie materiału do analizy, przygotowanie manuskryptu.

Mój sumaryczny udział w publikacji wynosił: 50%

2. **HAQUE, E.**; Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines. Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Konceptualizacja, metodologia, walidacja, badanie, gromadzenie i przygotowanie materiału do analizy, przygotowanie manuskryptu.

Mój sumaryczny udział w publikacji wynosił: 57%

3. **HAQUE, E.**; Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Konceptualizacja i pisanie rękopisów, projektowanie postaci.

Mój sumaryczny udział w publikacji wynosił: 55%

Jastrzebiec 15/03/22
Data i miejsce

Effi Haque
podpis

Effi Haque
Department of Experimental Embryology
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Statement

I hereby declare that in the publications constituting the basis of my doctoral dissertation, I had a leading contribution

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks**. Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Conceptualization, methodology, validation, investigation, collection and preparation of material for analysis, manuscript preparation.

My total share in the publication was: 50%

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Conceptualization, methodology, validation, investigation, collection and preparation of material for analysis, manuscript preparation.

My total share in the publication was: 57%

3. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis**. Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Conceptualization and manuscript writing, figure design.

My total share in the publication was: 55%

Jastrzebiec
Date and place 15/03/22

Effi Haque
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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Pomoc w napisaniu publikacji: Konceptualizacja, metodologia, walidacja, badanie, kuracja danych, pisanie oryginalnego przygotowania projektu, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 17

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Pomoc w napisaniu publikacji: Konceptualizacja, metodologia, walidacja, badanie, kuracja danych, pisanie oryginalnego przygotowania projektu, przegląd i edycja.

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Pomoc w napisaniu publikacji: Konceptualizacja, pisanie oryginalnego projektu, przygotowanie, recenzja i edycja.

Mój sumaryczny udział w publikacji wynosił: 17

Data i miejsce

2022 Feb 28

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

   Assistance in writing publication: Conceptualization, methodology, validation, investigation, data curation, writing original draft preparation, review and editing.

   My total share in the publication was: 17

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

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   Assistance in writing publication: Conceptualization, writing original draft preparation, review and editing

   My total share in the publication was: 17

Date and place

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Pomoc w napisaniu publikacji: Przegląd i edycja

Mój sumaryczny udział w publikacji wynosił: 4%

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Pomoc w napisaniu publikacji: Przegląd i edycja

Mój sumaryczny udział w publikacji wynosił: 4%

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

17.03.2022

Date and place

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Oświadczenie

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Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

2. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

Date and place

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.**; Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Pomoc w napisaniu publikacji: Badanie, kuracja danych, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 5%

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.**; Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Assistance in writing publication: Investigation, data curation, review and editing.

My total share in the publication was: 5%

Date and place

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Kyoto, Japan

Signature

Masahiko Taguchi

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.**; Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Pomoc w napisaniu publikacji: Badanie, kuracja danych, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 5%

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Shun Sakuraba

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Assistance in writing publication: Investigation, data curation, review and editing.

My total share in the publication was: 5%

Date and place

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Shun Sakuraba

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

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Pomoc w napisaniu publikacji: Kuracja danych, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 5%

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Pomoc w napisaniu publikacji: Badanie, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 5%

Data i miejsce

7 March, 2022.
Kyoto, Japan

podpis

河野秀俊

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Data curation, review and editing.

My total share in the publication was: 5%

2. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Assistance in writing publication: Investigation, review and editing.

My total share in the publication was: 5%

Date and place

7 March, 2022
Kyoto, Japan

Signature

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Instytut Genetyki i Biotechnologii Zwierząt PAN

Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

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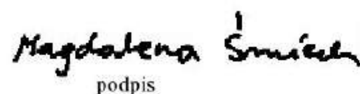
   Pomoc w napisaniu publikacji: Przegląd i edycja.

   Mój sumaryczny udział w publikacji wynosił: 4%

04.03.2022, Warszawa

Data i miejsce



podpis

Magdalena Śmiech (PhD)
Laboratory of Genome Editing and Transcriptional Regulation
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Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Assistance in writing publication: Investigation, review and editing

My total share in the publication was: 5%

2. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing

My total share in the publication was: 4%

04.03.2022, Warszawa

Date and place

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.;** Teeli, A.S.; Winiarczyk, D.; Taguchi, M.; Sakuraba, S.; Kono, H.; Leszczyński, P.; Pierzchała, M.; Taniguchi, H. **HNF1A POU Domain Mutations Found in Japanese Liver Cancer Patients Cause Downregulation of HNF4A Promoter Activity with Possible Disruption in Transcription Networks.** Genes 2022, 13, 413. https://doi.org/10.3390/genes13030413

Pomoc w napisaniu publikacji: Metodologia, walidacja, badanie, przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 5%

2. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

04-03-2022; Kashmir, INDIA.

Data i miejsce — podpis

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Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Methodology, validation, investigation, review and editing.

My total share in the publication was: 5%

2. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

04-03-2022; Kashmir, INDIA

Date and place | Signature

Marie France Bouchard (PhD)
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Centre de Recherche du CHU de Québec-Université Laval and Centre de Recherche en Reproduction, Développement et Santé Intergénérationnelle (CRDSI), Quebec, QC GIV4G2, Canada.

Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.;** Śmiech, M.; Łuczyńska, K.; Bouchard, M.F.; Viger, R.; Kono, H.; Pierzchała, M.; Taniguchi, H. **NRF2 DLG Domain Mutations Identified in Japanese Liver Cancer Patients Affect the Transcriptional Activity in HCC Cell Lines.** Int. J. Mol. Sci. 2021, 22, 5296. https://doi.org/10.3390/ijms22105296

Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

2022/03/04, Québec

Data i miejsce podpis

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Statement

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Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

2022/03/04, Québec

Date and place

Signature

Prof. Dr. hab. Atanas G Atanasov
Instytut Genetyki i Biotechnologii Zwierząt PAN

Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

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Pomoc w napisaniu publikacji: Przeglądanie i edytowanie.

Mój sumaryczny udział w publikacji wynosił: 4%

Data i miejsce: Vienna 04.03.2022

podpis

Prof. Dr. hab. Atanas G Atanasov
Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing

My total share in the publication was: 4%

Date and place: Vienna 04.03.2022

Signature

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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

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Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

04/03/2022
Data i miejsce

podpis

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Department of Translational Stem Cell Biology, Research Institute of the Medical University of Varna, 9002 Varna, Bulgaria

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

04/03/2022

Date and place

Signature

M Rezaul Karim (PhD)
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Oświadczenie

Niniejszym oświadczam, że mój udział w publikacji stanowiącej podstawę rozprawy doktorskiej mgr Effi Haque był następujący:

1. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

09/03/22

Data i miejsce

Rezaul Karim

podpis

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Department of Biotechnology and Genetic Engineering
Jahangirnagar University, Savar, Dhaka 1342, Bangladesh

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

1. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing.

My total share in the publication was: 4%

09/03/2022

Date and place

Rezaul Karim

Signature

Robert Viger (PhD)
Reproduction, Mother and Child Health, Centre de Recherche du CHU de Québec-Université Laval and Centre de Recherche en Reproduction, Développement et Santé Intergénérationnelle (CRDSI), Quebec, QC GIV4G2, Canada.

Oświadczenie

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Pomoc w napisaniu publikacji: Przegląd i edycja.

Mój sumaryczny udział w publikacji wynosił: 4%

Data i miejsce March 4, 2022 at Quebec, Canada

podpis:

Prof. Robert Viger (PhD)
Reproduction, Mother and Child Health, Centre de Recherche du CHU de Québec-Université Laval and Centre de Recherche en Reproduction, Développement et Santé Intergénérationnelle (CRDSI), Quebec, QC GIV4G2, Canada.

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Assistance in writing publication: Review and editing

My total share in the publication was: 4%

Date and place March 4, 2022 at Quebec, Canada

Signature

Dawid Winiarczyk (PhD)
Zakład Embriologii Doświadczalnej
Instytut Genetyki i Biotechnologii Zwierząt PAN

Oświadczenie

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Pomoc w napisaniu publikacji: Przeglądanie i edytowanie.

Mój sumaryczny udział w publikacji wynosił: 5%

2. **HAQUE, E.;** Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. **Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis.** Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Pomoc w napisaniu publikacji: Przeglądanie i edytowanie.

Mój sumaryczny udział w publikacji wynosił: 4%

Data i miejsce
1.03.22, Jastrzębiec

podpis
Winiarczyk

Dawid Winiarczyk (PhD)
Department of Experimental Embryology
Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Review and editing

My total share in the publication was: 5%

2. HAQUE, E.; Karim, M.R.; Salam Teeli, A.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant NRF2 Activation-Mediated Transcription Networks: Interaction of NRF2-KEAP1 Controls the Fate of Hepatocarcinogenesis. Int. J. Mol. Sci. 2020, 21, 5378. https://doi.org/10.3390/ijms21155378

Assistance in writing publication: Review and editing

My total share in the publication was: 4%

Date and place
7.03.22, Jastrzębiec

Signature
Winiarczyk

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Laboratorium Edycji Genomu i Regulacji transkrypcji
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Instytut Genetyki i Biotechnologii Zwierząt PAN

Oświadczenie

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Pomoc w napisaniu publikacji: Przegląd i edycja
Mój sumaryczny udział w publikacji wynosił: 4%

Data i miejsce | **podpis**

18. 03. 22, Jastrzębiec | Kamila Łuczyńska

Kamila Łuczyńska, M.D.
Laboratory of Genome Editing and Transcriptional Regulation
Department of Experimental Embryology
Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences

Statement

I declare that my contribution in the publication which is the basis for the doctoral dissertation of Effi Haque, MA was as follows:

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Assistance in writing publication: Review and Editing

My total share in the publication was: 4%

Date and place

18.03.22, Jastrzębiec

Signature

Kamila Łuczyńska