



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Keap1-Nrf2 signalling in pancreatic cancer

Citation for published version:

Hayes, AJ, Skouras, C, Haugk, B & Charnley, RM 2015, 'Keap1-Nrf2 signalling in pancreatic cancer', *International Journal of Biochemistry and Cell Biology*, vol. 65, pp. 288-299.
<https://doi.org/10.1016/j.biocel.2015.06.017>

Digital Object Identifier (DOI):

[10.1016/j.biocel.2015.06.017](https://doi.org/10.1016/j.biocel.2015.06.017)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

International Journal of Biochemistry and Cell Biology

Publisher Rights Statement:

Author's final peer-reviewed manuscript as accepted for publication

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.





Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biociel

Review

Keap1–Nrf2 signalling in pancreatic cancer

Alastair J. Hayes^{a,*}, Christos Skouras^b, Beate Haugk^c, Richard M. Charnley^d^a MRC Centre for Inflammation Research, University of Edinburgh, Queen's Medical Research Centre, Room C2.18, 47 Little France Crescent, Edinburgh, Scotland EH16 4TJ, United Kingdom^b School of Clinical Surgery, College of Medicine and Veterinary Medicine, University of Edinburgh, Room SU 305, Chancellor's Building, 49 Little France Crescent, Edinburgh, Scotland EH16 4SB, United Kingdom^c Department of Cellular Pathology, Newcastle upon Tyne Hospitals NHS Foundation Trust, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, England NE1 4LP, United Kingdom^d Department of Hepato-Pancreatico-Biliary Surgery, Newcastle upon Tyne Hospitals NHS Foundation Trust, Freeman Hospital, High Heaton, Newcastle upon Tyne, England NE7 7DN, United Kingdom

ARTICLE INFO

Article history:

Received 24 April 2015

Received in revised form 21 June 2015

Accepted 22 June 2015

Available online 24 June 2015

Keywords:

Nrf2

Keap1

NQO1

AKR1B10

Pancreatic cancer

ABSTRACT

Transcription factor NF-E2 p45-related factor 2 (Nrf2, also called Nfe2l2), a master regulator of redox homeostasis, and its dominant negative regulator, Kelch-like ECH-associated protein 1 (Keap1), together tightly control the expression of numerous detoxifying and antioxidant genes. Nrf2 and the 'antioxidant response element' (ARE)-driven genes it controls are frequently upregulated in pancreatic cancer and correlate with poor survival. Upregulation of Nrf2 is, at least in part, *K-Ras* oncogene-driven and contributes to pancreatic cancer proliferation and chemoresistance. In this review, we aim to provide an overview of Keap1–Nrf2 signalling as it relates to pancreatic cancer, discussing the effects of inhibiting Nrf2 or Nrf2/ARE effector proteins to increase chemosensitivity.

© 2015 Elsevier Ltd. All rights reserved.

Contents

1. Introduction	289
2. The Keap1–Nrf2–ARE pathway	289
2.1. Redox cellular stress	289
2.2. Cytoprotection by ARE-driven genes	289
2.3. Endogenous Nrf2 upregulatory mechanisms	290
3. Dysregulation of Keap1–Nrf2 signalling in pancreatic cancer	291
3.1. ARE-driven gene expression is increased in pancreatic cancer	291
3.2. Nrf2 expression is higher than Keap1 and correlates with poor survival	291
3.3. Absence of <i>KEAP1</i> or <i>NFE2L2</i> gene mutations in pancreatic cancer	291
3.4. Increased Nrf2 activity in pancreatic carcinogenesis	291
3.5. Variable Nrf2, Keap1 and ARE-target gene expression across PDAC cell lines	292
3.6. The effects of suppressing Nrf2 compared to Nrf2/ARE effector proteins in PDAC	292
3.6.1. PDAC cell viability, chemosensitivity and proteasome activity with Nrf2 inhibition	293
3.6.2. PDAC cell viability and chemosensitivity with suppression of Nrf2/ARE downstream effector proteins	293

Abbreviations: AKR1B10, Aldo–keto reductase family 1 b10; ARE, antioxidant response element; Bach1, BTB and CNC homology 1; CNC, Cap 'n' collar; Cul3, Cullin-3; ER, endoplasmic reticulum; GCLC, glutamate–cysteine ligase catalytic; HO-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; MRPs, multi-drug resistance associated protein transporters; Nfe2l2, nuclear factor (erythroid-derived 2)-like 2; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor E2 p45-related factor 2; PDAC, pancreatic ductal adenocarcinoma; PERK, PKR-like ER kinase; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TP53INP1, tumor protein p53-induced nuclear protein 1.

* Corresponding author. Tel.: +44 0131 242 6593.

E-mail addresses: alastair.hayes@ed.ac.uk (A.J. Hayes), christos.skouras@ed.ac.uk (C. Skouras), beate.haugk@nuth.nhs.uk (B. Haugk), richard.chnley@nuth.nhs.uk (R.M. Charnley).

<http://dx.doi.org/10.1016/j.biociel.2015.06.017>

1357-2725/© 2015 Elsevier Ltd. All rights reserved.

3.7. Nrf2 inhibitor therapy for pancreatic cancer	294
3.7.1. Nrf2 inhibitors.....	294
3.7.2. Challenges in the application of Nrf2 inhibitor therapy for pancreatic cancer	294
4. Conclusions.....	295
Authors' contribution	295
Acknowledgements	295
Appendix A.....	295
References.....	297

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with the lowest survival rates and worst prognosis of all gastrointestinal cancers. The tumour has a prominent stromal component and is intrinsically chemoresistant. Additionally, PDAC characteristically disseminates early in the clinical course, when there are minimal symptoms, and the diagnosis is often reached at an advanced stage of the disease. Surgical resection with or without adjuvant chemotherapy can be indicated in localised disease, but unfortunately recurrence is common due to the particularly aggressive biology of this cancer.

The development of neoplastic disease, not least PDAC, is a complex multistep process involving a variety of aberrant signalling pathways which can reprogram energy metabolism, promote a growth-permissive tumour microenvironment that helps sustain proliferative signalling and replicative immortality, activate local tissue invasion and distant metastasis (Hanahan and Weinberg, 2011). Somatic gene mutations show particular heterogeneity in pancreatic cancer, and it is this understanding of the disease, in the context of the limited benefits seen in PDAC clinical trials, that underpins the increasing shift towards biology-tailored, personalised drug therapy (Chang et al., 2014). Urgent advances are required to further unlock the complex biological mechanisms that contribute to pancreatic cancer chemoresistance which might lead to tractable drug targets.

Transcription factor *nuclear factor erythroid 2 p45-related factor 2* (Nrf2, also called Nfe2l2) is known not only for its role in cancer chemoprevention but, conversely, also in cancer cell chemoresistance (Lau et al., 2008). Nrf2 forms part of the Keap1–Nrf2–ARE oxidative stress response pathway, and has recently been identified as a contributory factor both in pancreatic carcinogenesis, and in chemoresistance against gemcitabine, amongst other anti-cancer cytotoxics. In the present article we review the current understanding of the Keap1–Nrf2–ARE pathway, with an overview of how this system can become dysregulated, with a particular focus on its relation to pancreatic cancer.

2. The Keap1–Nrf2–ARE pathway

Nrf2 provides one of the most versatile mechanisms of adaptation to cellular oxidative stress. It is a master regulator of redox homeostasis, and is widely expressed in human tissues, particularly those continuously exposed to environmental toxins such as the skin, lungs and the upper digestive tract. Together with NF-E2 p45, Nrf1, Nrf3, Bach1 and Bach2, Nrf2 is a member of the cap 'n' collar (CNC) family of transcription factors which share a common basic-region leucine zipper (bZIP) structure that exists in its Neh1 domain (Zhang, 2006). The abundance of Nrf2 within the cell is normally tightly restricted by a redox-sensitive E3 ubiquitin ligase substrate adaptor called Keap1 (*Kelch-like ECH-associated protein 1*). This regulator was originally identified by yeast two-hybrid analysis (Itoh et al., 1999), and was confirmed as a repressor by the finding that Nrf2 accumulated in the nucleus and Nrf2-target genes were upregulated in *Keap1* gene knockout mice (Wakabayashi et al.,

2003). Moreover, knockdown of Keap1 in human cell lines similarly increases expression of Nrf2-target genes (Devling et al., 2005; Agyeman et al., 2012). In the cytoplasm, Keap1 may be associated with the cytoskeleton and interacts with cullin-3 (*via* its BTB domain) and the Neh2 domain of Nrf2 (*via* its Kelch-repeat domain) (McMahon et al., 2003; Nioi and Hayes, 2004). In doing so, Keap1 facilitates Cul3-mediated ubiquitination of Nrf2 and subsequent proteolytic destruction of the CNC-bZIP transcription factor by the 26S proteasome (Itoh et al., 1999; Zhang et al., 2004). Under normal homeostatic conditions, when ubiquitination is unimpeded, Nrf2 has a short half-life of approximately 10–30 min (Itoh et al., 2003). Cysteine residues present in Keap1 function as redox sensors that when modified by oxidation or adduction by electrophiles, cause loss of Nrf2 ubiquitination. Once modified and inactive, the fate of Keap1 is controversial, with a few authors suggesting that Nrf2 is liberated from Keap1 (Itoh et al., 2003), while others suggest that this does not occur (McMahon et al., 2006; Baird et al., 2013). In any event, upon inactivation of Keap1, newly translated Nrf2 translocates to the nucleus where it heterodimerizes with small Maf (sMaf) proteins before it binds DNA (Motohashi and Yamamoto, 2004). Together, the Nrf2-sMaf protein heterodimer binds to a specific DNA sequence, called the antioxidant response element (ARE), in the promoter region of target genes (Itoh et al., 1997; Zhang, 2006). By this mechanism, Nrf2 constitutes a unique redox regulator whose activity can be increased in response to imbalances in normal redox caused by oxidative and electrophilic stressors (Fig. A1).

2.1. Redox cellular stress

While living cells operate optimally within certain pH and temperature limits, they also require optimal redox balance for metabolic processes, and their ability to adapt rapidly to perturbations in homeostasis is essential for survival. Cells can be threatened by reactive oxygen species (ROS) and by toxic secondary metabolites generated from ROS-mediated cell damage, giving rise to oxidative stress (Halliwell and Gutteridge, 2007). The latter involves various deleterious processes that result from an imbalance between antioxidant defences and excessive ROS (Turrens, 2003; Acharya et al., 2010). Resulting damage from oxidative stress, if substantial, can lead to cell death unless adaptive processes are implemented that can withstand the harmful effects. ROS comprises oxygen-derived molecules and free radicals which can arise during normal metabolism (Turrens, 2003), and also during cell proliferation or abnormal and stressful events, such as endoplasmic reticulum (ER) stress or oncogenic transformation (Zhang and Kaufman, 2008; Logsdon and Ji, 2013; Kong et al., 2013). Moreover, exogenous toxic environmental agents, such as chemotherapy drugs and cigarette combustion products, may also induce free radicals (Hayes and McMahon, 2009; Ju et al., 2015).

2.2. Cytoprotection by ARE-driven genes

Upon translocation to the nucleus, Nrf2 can activate the transcription of approximately 200 genes (Hayes and Dinkova-Kostova, 2014). The coordinated activation of target genes by Nrf2 is

possible because of specific antioxidant response element (ARE) sequences within each gene regulatory region, and these genes encode for enzymes that contribute a wide range of cytoprotective functions including regulation of antioxidant and drug detoxification systems, NADPH regeneration, modulation of intermediary metabolism, and regulation of the proteasome (see Fig. A2). Normal cells rely on the Keap1–Nrf2–ARE pathway to handle redox stress and defend against cellular damage. Indeed, Nrf2/ARE can be activated by relatively non-toxic dietary compounds, such as isothiocyanates, coumarins and phenolic antioxidants, which protects against subsequent exposure to harmful xenobiotics (Hayes et al., 2000; Higgins et al., 2009; McWalter et al., 2004). For example, pre-treatment of fibroblasts with the isothiocyanate sulforaphane, produced by hydrolysis of glucoraphanin (present in cruciferous vegetables such as broccoli), elicits an Nrf2-mediated adaptive response that markedly increases intracellular levels of glutathione (Hayes et al., 2008; Higgins et al., 2009). A brief overview of the major Nrf2/ARE target genes and Nrf2 effector proteins is described, with a focus on those known to be upregulated in PDAC.

The glutathione and thioredoxin systems are the two major thiol-dependent endogenous antioxidant systems found in mammalian cells which scavenge reactive oxygen and nitrogen species (Lu and Holmgren, 2014). Nrf2/ARE tightly regulate glutathione by controlling the expression of the glutamate–cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits that combine to form a heterodimer which catalyses the rate-limiting step in glutathione biosynthesis. Nrf2/ARE also control the expression of glutathione peroxidase (*GPX2* gene) and glutathione reductase (*GSR1* gene), which together ensure intracellular levels of peroxides are reduced and, using NADPH as a cofactor, oxidised glutathione (GSSG) is returned to its reduced (GSH) state. Nrf2/ARE signalling also controls the expression of cytosolic thioredoxin (*TXN1* gene), thioredoxin reductase (*TXNRD1* gene), and sulfiredoxin (*SRXN1* gene), all of which reduce oxidised protein thiols (Lu and Holmgren, 2014). In PDAC, the glutathione-based antioxidant system contributes to gemcitabine resistance (Kim et al., 2014), and the thioredoxin-based system supports cancer cell survival (Yan et al., 2009).

Cellular elimination of xenobiotics is another important function of Nrf2/ARE-mediated signalling which occurs through the transport of exogenous compounds out of the cell by multi-drug-resistance-associated protein transporters (MRPs), or else by intracellular drug metabolism. Expression of drug efflux transporter genes is dysregulated in PDAC and thought to contribute to chemoresistance by increased movement of anti-cancer drugs out of the cancer cell (Mohelnikova-Duchonova et al., 2013). Members of the ATP-binding cassette subfamily C (e.g. *ABCC1*, *ABCC3* and *ABCC5* genes) are under Nrf2/ARE-mediated regulation, and increased expression of these transporters in PDAC is associated with a poor treatment response (Mohelnikova-Duchonova et al., 2013). The basal and inducible expression of several phase I and phase II drug detoxification enzymes by the Nrf2/ARE system is widely recognised, and over expression of aldo–keto reductase (Chung et al., 2012; Li et al., 2013; Zhang et al., 2014; Yi and Oh, 2015), aldehyde dehydrogenase (Yi and Oh, 2015; Singh et al., 2015), and NQO1 (Awadallah et al., 2008) have been identified in several PDAC cell lines. Many of these drug-metabolizing enzymes as well as the antioxidant systems require NADPH as a cofactor, and Nrf2 regulates all four of the NADPH-generating enzymes. Thus, Nrf2 activity ensures that the expression of antioxidant and detoxification enzymes is coupled with a supply of the necessary cofactors. Moreover, whilst NADPH is required for the biosynthesis of lipids and nucleotides, Nrf2 also regulates critical enzymes in major metabolic pathways, such as the pentose phosphate pathway (PPP) and fatty acid synthesis, and in doing so

influences the biosynthesis of carbohydrates, lipids, nucleic acids, and amino acids (for a detailed review see ‘Hayes and Dinkova-Kostova, 2014’). In the context of oncogenic transformation and proliferation, wherein cancer cells consume large quantities of nutrients and maintain high levels of anabolism, Nrf2/ARE signalling has been shown to redirect glucose and glutamine into anabolic pathways (Mitsuishi et al., 2012) and in this way could support cell proliferation in pancreatic carcinogenesis. Of particular importance to PDAC, the PPP has been found to be an essential fuel source for rapid K-Ras-induced cell proliferation (Weinberg et al., 2010).

Heme oxygenase-1 (HO-1), which catalyzes the first and rate-limiting enzymatic step of heme degradation, is another important cytoprotective enzyme encoded by an ARE-target gene with antioxidant, antiapoptotic, proliferative and pro-angiogenic properties (Dunn et al., 2014). In human PDAC tissue, HO-1 is upregulated (Berberat et al., 2005), and is discussed in more detail later. Another function of Nrf2/ARE-driven signalling is to increase proteasomal gene expression (e.g. *s5a/psmd4* and *alpha5/psma5*). Proteasomal activity is important for the removal of damaged and misfolded proteins that otherwise could impair cell function (Kwak et al., 2003). Nrf2 inhibition in tumour cells down-regulates the proteasome, rendering such cells more susceptible to apoptosis (Arlt et al., 2009). Lastly, evidence also exists that Nrf2 combats ER stress (Meakin et al., 2014), possibly by orchestrating adaption to oxidative stress (Cullinan and Diehl, 2004), and since correct processing and secretion proteins is an important function of the exocrine and endocrine pancreas, this may be an important contribution of the CNC–bZIP transcription factor for pancreatic function in both health and disease (Sah et al., 2014).

2.3. Endogenous Nrf2 upregulatory mechanisms

Whilst Nrf2 activity is usually tightly controlled, there are several endogenous ways by which Nrf2 activity may be altered. Firstly, *KEAP1* epigenetic silencing by hypermethylation of the promoter gives rise to Nrf2 upregulation (Wang et al., 2008; Zhang et al., 2010; Muscarella et al., 2011). Similarly, and by way of contrast, epigenetic alterations to the *NFE2L2* promoter, such as promoter hypermethylation or single nucleotide polymorphisms, have been found to substantially repress Nrf2 transcription and activity (Yu et al., 2010; Suzuki et al., 2013). Direct mutations of the *NFE2L2* gene resulting in modification of those residues in Nrf2 that interact with Keap1, permanently increase the activity of the CNC–bZIP transcription factor (Hayes and McMahon, 2009). Functional *KEAP1* mutations (i.e. missense, frameshift and homozygous deletion of *KEAP1*) which arise in a number of cancers (i.e. lung, gallbladder, breast), result in up-regulated Nrf2–ARE gene transcription (Singh et al., 2006; Shibata et al., 2008). There is an extensive and increasing list of factors known to trans-activate the *NFE2L2* gene leading to overexpression of Nrf2 (Hayes and Dinkova-Kostova, 2014); amongst these are oncogenes important in pancreatic cancer, which will be discussed in more detail later. Beyond translation, Keap1 is silenced through mechanisms such as microRNA repression (i.e. miR-141, miR-200a) (Eades et al., 2011), or autophagosome regulation (i.e. p62/SQSTM1) (Taguchi et al., 2012), resulting in ineffective Nrf2 repression. Post-translational modifications of Keap1 can inhibit its association with the Cul3 ubiquitin ligase, causing Nrf2 saturation of Keap1, thereby allowing nuclear import of newly formed Nrf2. More specifically, alterations to particular Keap1 cysteine residues (e.g. Cys-273, Cys-288 and Cys-151) which act as sensors for electrophiles have been identified as key functional sites which, when modified, block Keap1-mediated ubiquitylation and turn-over of Nrf2, as has the formation of a disulphide bond between residues Cys-226 and

Cys-613 (Wakabayashi et al., 2004; Zhang and Hannink, 2003; Fourquet et al., 2010; Hourihan et al., 2013).

Other cytoplasmic proteins may also interfere with Keap1–Nrf2 interaction. Indeed, there exist several proteins (e.g. PALB2, PGAM5, WTX) that are known to bind the Kelch domain of Keap1, preventing Keap1–Nrf2 association (Hayes and Dinkova-Kostova, 2014). Additionally, Keap1 is not the only negative regulator of Nrf2, and other proteins (e.g. β -TrCP) can mediate Nrf2 ubiquitination through other binding sites on Nrf2 (Rada et al., 2011; Chowdhry et al., 2013). Finally, phosphorylation can prevent Keap1–Nrf2 interaction and thereby increase Nrf2 nuclear translocation (Zipper and Mulcahy, 2000). For example, during ER stress PERK phosphorylates Nrf2 which evades proteasomal destruction and translocates to the nucleus, and thereby switching on cytoprotective ARE-driven genes (Cullinan et al., 2003).

3. Dysregulation of Keap1–Nrf2 signalling in pancreatic cancer

The Keap1–Nrf2–ARE pathway is vital in protecting normal cells from oxidative stress, but in cells harbouring activated oncogenes, upregulation of the ARE-gene battery may offer survival and proliferative advantages within the metabolic cancer microenvironment, and help withstand attack from immune regulation or cytotoxic chemotherapy compounds (Hayes and McMahon, 2009; Lau et al., 2008; Homma et al., 2009). Several forms of cancer, including lung (Padmanabhan et al., 2006) and endometrial cancers (Chen et al., 2010), appear to hijack the defence provided by ARE-driven genes through constitutive upregulation of Nrf2, and there is growing evidence to support an important role for the Keap1–Nrf2–ARE pathway in pancreatic cancer. A review of the literature investigating Nrf2 signalling in PDAC is discussed below, and summarised in Fig. A3.

3.1. ARE-driven gene expression is increased in pancreatic cancer

Evidence for the contributory role of the Nrf2–ARE system in PDAC appears to have first emerged from the study of cell lines and clinical samples, of which the latter have consistently shown moderate to strong immunohistochemical staining of malignant epithelium for proteins regulated via ARE sequences in their gene promoters. A prototypical Nrf2 downstream protein, NQO1 (*NAD(P)H:quinone oxidoreductase 1*), was found to be over-expressed ten-fold compared to normal human pancreatic tissue (Logsdon et al., 2003). Strong immunohistochemical staining for NQO1 has also been demonstrated in premalignant pancreatic dysplastic lesions, known as Pancreatic Intraepithelial Neoplasias (PanINs), suggesting that it is frequently activated early in pancreatic carcinogenesis (Awadallah et al., 2008; Lewis et al., 2005). However, specificity for PDAC may be poor since NQO1 is also over-expressed in non-tumorous pancreatic specimens from smokers, and in pancreatitis tissue (Lewis et al., 2005; Lyn-Cook et al., 2006). There does not appear to be an increase in NQO1 gene polymorphisms in pancreatic cancer resections compared to normal pancreatic tissue (Lyn-Cook et al., 2006). DeNicola et al. demonstrated that Nrf2-deficient murine PanINs were negative for Nqo1 in an *Nfe2l2* gene knockout model, supporting the notion that Nrf2 plays a dominant role in upregulating the protein in pancreatic cancer (DeNicola et al., 2011). Likewise, another Nrf2–ARE downstream protein, AKR1B10 (*aldo-keto reductase family 1 member b10*), has been found to be over-expressed in PDAC, and also in the majority of PanIN lesions. Amongst well- and moderately differentiated pancreatic cancer specimens, approximately 70 per cent were found to over-express AKR1B10, compared to surrounding

morphologically normal ducts (Chung et al., 2012). This Nrf2-target protein is of particular interest because its inhibition both impedes pancreatic carcinogenesis, and leads to a reduction in activated K-Ras protein (Li et al., 2013).

3.2. Nrf2 expression is higher than Keap1 and correlates with poor survival

Several authors have demonstrated a direct increase of Nrf2 protein levels in human pancreatic cancer tissue, particularly in the cytoplasm of the malignant epithelial cells (DeNicola et al., 2011; Hong et al., 2010; Lister et al., 2011). Specifically, Lister et al. found that the majority of pancreatic cancer specimens (48 of 57 specimens) showed strong Nrf2 cytoplasmic staining by immunocytochemistry, compared to the relatively infrequent strong staining amongst benign ducts (four of 21 specimens). No difference in nuclear staining was detected between cancers and benign ducts (Lister et al., 2011). Hong et al. similarly found frequent strong cytoplasmic Nrf2 staining in pancreatic cancer specimens, but in contrast to Lister et al., a higher proportion of nuclear staining was detected in PDAC compared to normal tissue. Importantly, following pancreatic resection, an increase in nuclear Nrf2 expression was found to correlate with a significant reduction in overall survival (Soini et al., 2014). Levels of cytoplasmic Keap1 are higher in human PDAC tissue than in benign ductal epithelium, but still only detectable in ~30%, suggesting that Keap1 transcription remains relatively low compared to the much higher Nrf2 levels (Lister et al., 2011).

3.3. Absence of KEAP1 or NFE2L2 gene mutations in pancreatic cancer

DeNicola et al. tested for somatic mutations in *KRAS*, *NFE2L2* and *KEAP1* genes by sequencing over 100 human pancreatic cancer resection specimens. The vast majority had *KRAS* mutations, in keeping with the literature. There were no *NFE2L2* mutations in this series and just three nonsynonymous *KEAP1* mutations (see supplementary Fig. 14b of paper by DeNicola et al.), giving a mutational prevalence of approximately one per cent, which is considerably lower than that observed in other cancers (DeNicola et al., 2011). In corroboration with this, a publically available transcript database that contained 24 PDAC cases revealed no *KEAP1* or *NFE2L2* mutations (Lister et al., 2011; Jones et al., 2008). Therefore, unlike biliary tract cancers that frequently contain functional *KEAP1* mutations (Shibata et al., 2008), over-expression of Nrf2 in pancreatic cancer does not appear to be a consequence of somatic gene mutations in *NFE2L2* or in *KEAP1*.

3.4. Increased Nrf2 activity in pancreatic carcinogenesis

During pancreatic carcinogenesis, Nrf2 activity is increased and this may also be important during oncogenic transformation in chronic pancreatitis.

Using a conditional transgenic *K-Ras* knock-in mouse PDAC model, Nrf2 deletion in these mice has been shown to result in fewer PanINs, reduced cellular proliferation, and reduced tumour burden, whilst at the same time maintaining elevated DNA oxidation (DeNicola et al., 2011). The findings of DeNicola et al. may at first appear paradoxical; that ROS, which are traditionally considered to contribute to carcinogenesis through mutagenic oxidation of DNA, are repressed in pancreatic carcinogenesis by activation of the ROS-detoxification program. The explanation, however, lies in a novel finding of oncogene-mediated Nrf2 activation. Firstly, DeNicola et al. demonstrated that expression of *K-Ras*, *B-Raf* and *Myc* oncogenic alleles in murine embryonic fibroblasts increased

Nfe2l2 transcription and thus Nrf2 activity in a Keap1-independent manner. This is not a universal feature of oncogenes, as demonstrated by the inability of activated *Notch 1* and β -Catenin to upregulate the Nrf2–ARE system (DeNicola et al., 2011). DeNicola et al. demonstrated that *K-Ras* and *B-Raf* stimulated the binding of Jun and Myc (of the MAP kinase signalling pathway) to the *Nfe2l2* gene promoter; thereby switching on the Nrf2–ARE system.

TP53INP1 (tumour protein 53-included protein 1) is a stress-induced p53 (protein 53) target gene that induces cell growth arrest and apoptosis by modulating p53 activity, and contributes to the p53-driven oxidative stress response. In human tissue, *TP53INP1* expression is found in normal pancreas and low grade dysplastic lesions (PanIN-1A), but only half of PanIN-2s express this protein and it is absent in high grade lesions (PanIN-3) and in PDAC, which suggests that expression of *TP53INP1* is typically lost early in pancreatic carcinogenesis (Gironella et al., 2007). Not only has loss of *TP53INP1* been shown to accelerate murine PanIN formation, this loss is associated with increased levels of Nrf2 (Al Saati et al., 2013). It is known that absent *TP53INP1* gives rise to diminished antioxidant activity, which can arise through both p53-dependent and p53-independent signalling mechanisms (Cano et al., 2009). Either way, it is likely that secondary increased intracellular ROS levels as a consequence of diminished *TP53INP1* results in oxidation of Keap1 residues thereby promoting nuclear translocation of Nrf2. There are, however, several points of crosstalk between p53 and Nrf2 signalling (Rotblat et al., 2012) which may provide an alternative mechanism for increased Nrf2 levels as a result of *TP53INP1* deficiency in pancreatic cancer.

It has long been recognised that chronic pancreatitis (CP), which is a destructive fibro-inflammatory disease of the pancreas, increases the risk of developing pancreatic cancer (Whitcomb et al., 2004). Indeed, CP patients have an overall 16-fold higher risk of developing PDAC compared to unaffected individuals (Rivera et al., 1997). Histologically, there are many features such as fibrosis, desmoplasia and immune cell infiltration that are observed in both PDAC and CP (Bai et al., 2011; Ino et al., 2013). The contribution of CP to PDAC is highlighted by the observation that activation of *K-Ras*^{G12V} in acinar/centroacinar cells in adult mice (mimicking the occurrence of activating *KRAS* mutations arising in adult humans) necessitates the induction of pancreatitis in order to induce PanIN lesions and cancer (Guerra et al., 2007; Zhang et al., 2013). Oxidative stress is a well recognised feature of CP which is evidenced by the findings of increased in lipid peroxidation products in the pancreatic juice and circulating plasma of CP patients (Ganesh et al., 1999; Stevens et al., 2012) and elevated pancreatic tissue DNA oxidation in rodent CP models (Zeki et al., 2002). Several mechanisms are known to contribute to the perturbed redox status found in CP, such as free radical production during alcohol metabolism (Altomare et al., 1996), ER stress (Sah et al., 2014) and ROS production by locally activated immune cells (O'Byrne and Dalglish, 2001). Within the pro-inflammatory state found in CP, increased expression of Nrf2 and phase II antioxidant enzymes (NQO1, HO-1) have been shown to be present, inferring cytoprotection (Yang et al., 2012). The problem appears to arise when oncogenic *K-Ras* is also present, as pancreatic tissue injury repair is compromised (Zhang et al., 2013). Pancreatitis in the context of oncogenic *K-Ras* drives progression of acinar-ductal metaplasia into progressive PanIN lesions, rather than normal re-differentiation into acinar cells (Zhang et al., 2013). PanIN progression appears to be augmented by Nrf2 activity, with failure to upregulate Nrf2 resulting in increased tumour cell death (Zhang et al., 2013; DeNicola et al., 2011). Interestingly, Zhang et al. suggest that oncogenic Nrf2 activation by *K-Ras* may be dependent on Interleukin-6, and that these factors, together with STAT3

activation (Lesina et al., 2011), are required for PanIN progression to PDAC (Zhang et al., 2013).

3.5. Variable Nrf2, Keap1 and ARE-target gene expression across PDAC cell lines

Across pancreatic cancer cell lines, there is variability in basal Nrf2 protein expression (see Table A1). Hong et al. found Nrf2 to be over-expressed in several PDAC cell lines (Panc-1, AsPC-1, Colo-357), when compared to normal human pancreatic duct epithelial cells (Hong et al., 2010). Surprisingly, other PDAC cell lines which also have mutant *KRAS*, namely MiaPaCa-2 or FAM-PAC, have low levels of basal Nrf2 protein (Lister et al., 2011). Additionally, reported basal Nrf2 protein levels in the Panc-1 cell line have been variable, as detected by immunoblot (Hong et al., 2010; Lister et al., 2011; Arlt et al., 2013). With regard to Keap1, basal levels were reported as consistently elevated across PDAC cell lines, with the exception of Suit-2 (Lister et al., 2011). Perhaps not unsurprisingly, there also exists variability in the ARE-target gene expression amongst PDAC cell lines. This is most clearly demonstrated when considering differences between Panc-1 and AsPC-1 cells. The former have elevated Nrf2 expression but low NQO1 and GCLC levels, whilst AsPC-1 cells have much lower Nrf2 levels but high NQO1 expression (Hong et al., 2010). Whilst NQO1 is commonly over-expressed in the majority of PDAC cell lines (BxPC3, Capan1, MiaPaCa-2, AsPC-1) (Cullen et al., 2003), Panc-1, is unusual in this regard as it contains homogenous expression of the *NQO1**2 polymorphism, resulting in repressed expression (Siegel et al., 2012). Nrf2 knockdown reduces NQO1 and GCLC in AsPC-1 cells, supporting the notion of Nrf2-driven expression of these target genes in this line (Duong et al., 2014). For a thorough investigation of the downstream effects of Nrf2 repression and activation in the AsPC-1 cell line, see a recent report by Yi and Oh, 2015. Taking these findings together, it appears that basal activity of the Nrf2–ARE system is commonly upregulated in different PDAC cell lines, and that differences in ARE-target protein expression may arise not solely from upstream Keap1/Nrf2 dysregulated activity, but also from silencing gene polymorphisms and additional factors outwith the Keap1–Nrf2 pathway.

3.6. The effects of suppressing Nrf2 compared to Nrf2/ARE effector proteins in PDAC

To study the effects of down regulating the Nrf2/ARE pathway in PDAC, some investigators have targeted individual enzymes that are members of the ARE-gene battery, whilst others have repressed Nrf2. Of course, with the latter approach, multiple cytoprotective and proteasomal genes would be affected given that the function of Nrf2 is to regulate transcription of the large portfolio of ARE-regulated genes. The studies reviewed here employed several techniques to suppress Nrf2 or ARE-target protein activity; the specific use of Nrf2 inhibitors in PDAC is discussed later. Proliferation and apoptosis of PDAC cell lines following Nrf2/ARE suppression, including resistance to chemotherapy compounds, have been the major outcomes reported, but proteasome activity has also been assessed in Nrf2 knockdown (Arlt et al., 2013). The effects of Nrf2/ARE inhibition in PDAC have been investigated both *in vitro* (Chung et al., 2012; Duong et al., 2014; Nolan et al., 2007; Reigan et al., 2007; Dehn et al., 2006; Lister et al., 2011) and *in vivo* (Duong et al., 2014; Dehn et al., 2006; Lewis et al., 2004; Li et al., 2011; Ough et al., 2005; Zhang et al., 2014). The majority of *in vivo* studies have used MiaPaca-2 xenografts, wherein cancer cells were injected into the spleen (Li et al., 2011) or subcutaneously (Duong et al., 2014; Dehn et al., 2006; Lewis et al., 2004; Ough et al., 2005). Less frequently, Colo-357,

Panc-1 and CD18 subcutaneous xenograft tumour models have also been studied (Arlt et al., 2013; Zhang et al., 2014). Given the possible confounding off-target effects of inhibitor compounds, comparisons between Nrf2 and ARE-target protein suppression in specific cell lines are confined to those studies where RNAi knockdown was employed, or else highly selective ARE-target protein inhibitors.

3.6.1. PDAC cell viability, chemosensitivity and proteasome activity with Nrf2 inhibition

Nrf2 knockdown has been found to consistently reduce viability of several PDAC cell lines, including AsPC-1, Suit-2, MiaPaCa-2 and FAMPAC cell lines (Lister et al., 2011; Duong et al., 2014). Using an alternative method to repress Nrf2, by PIK-75 treatment to increase proteasomal Nrf2 degradation, increased apoptosis of AsPC-1 and MiaPaCa-2 cells was similarly observed (Duong et al., 2014).

As for Nrf2-dependent chemoresistance, reports have been variable across different PDAC cell lines. Enhanced sensitivity of AsPC-1 cells treated with cisplatin, phenethyl isothiocyanate and gemcitabine has been observed following Nrf2 knockdown (Ju et al., 2015; Hong et al., 2010; Duong et al., 2014). Similarly, knockdown of Nrf2 in Colo-357 cells resulted in reduced viability after treatment with cisplatin and phenethyl isothiocyanate (Hong et al., 2010). Up-regulation of Nrf2 by t-BHQ in AsPC-1 and Colo-357 cells undergoing cisplatin and camptothecin treatment increased cell survival, and also highlights the protective role of Nrf2 (Hong et al., 2010). In Suit-2 cells subjected to Nrf2 knockdown, enhanced sensitivity to cisplatin and gemcitabine was identified, but no discernable change in sensitivity following 5-FU treatment (Lister et al., 2011). Like Suit-2 cells, enhanced sensitivity to cisplatin was observed in FAMPAC cells with Nrf2 knockdown, but contrary to the findings in Suit-2, FAMPAC cells are found to have increased sensitivity to 5-FU but minimal response to gemcitabine sensitivity following knockdown (Lister et al., 2011). In Panc-1 cells, Nrf2 suppression enhanced the cytotoxic effects of etoposide (Arlt et al., 2013). Depletion of Nrf2 has been demonstrated to increase MiaPaCa-2 sensitivity to gemcitabine *in vitro* and *in vivo*, and limits the ability of these cells to adapt to gemtamicin-induced ROS formation (Ju et al., 2015; Duong et al., 2014).

Proteasome activity is consistently reduced following Nrf2 knockdown across PDAC cell lines (Arlt et al., 2013). This aspect of Nrf2 signalling is important for tumorigenesis as it provides anti-apoptotic protection and efficient clearance of irregular proteins.

3.6.2. PDAC cell viability and chemosensitivity with suppression of Nrf2/ARE downstream effector proteins

3.6.2.1. NAD(P)H:quinone oxidoreductase 1. The prototypical ARE-target gene protein, NQO1, is a drug-metabolising enzyme primarily located in the cytosol where it protects against ROS and directly metabolises highly toxic and reactive quinone species, as well as having a role in stabilising p53 (Ross et al., 2000; Nioi and Hayes, 2004). Quinones are oxidised aromatic compound derivatives, and exogenous sources include vehicle exhaust fumes and cigarette smoke. Inhibition of NQO1 *in vitro* was reported to suppress cell viability and the colony-forming capacity of BxPC-3 (Dehn et al., 2006) and MiaPaCa-2 cells (Cullen et al., 2003; Dehn et al., 2006; Li et al., 2011) in a dose dependent manner, using NQO1 inhibitors Dicumarol and 2-methyl indolequinone ES936. The delivery of NQO1 inhibitors either directly (intra-tumoral) (Lewis et al., 2004) or systemically (intra-peritoneal) (Dehn et al., 2006) appears to suppress growth of MiaPaCa-2 xenograft tumours *in vivo*. The dependency of NQO1 in the reported anti-proliferative effects of Dicumarol was brought into question as the principle cytotoxicity has since been attributed to off-target effects that are independent NQO1 (Nolan et al., 2007; Reigan et al., 2007).

However, the more specific, mechanism-based inhibitor of NQO1, ES936, inhibits proliferation of MiaPaCa-2 cells *in vitro* and *in vivo*, and thereby helps supports the contributory role of NQO1 in PDAC proliferation (Dehn et al., 2006).

As for chemoresistance, the anti-cancer activity of a naturally occurring quinone, β -Lapachone, which is an NQO1 substrate that is recognised as having antineoplastic properties, was ameliorated by NQO1 knockdown in MiaPaCa-2 cells (Ough et al., 2005). Additionally, Lewis et al. observed a reduction in the cytotoxic effects with treatment with the quinone streptonigrin to MiaPaCa-2 cells after Dicumarol, a rescue effect that was absent in CaCo-2 cells; the latter being a colon cancer cell line with low basal NQO1 expression (Lewis et al., 2005; Siegel et al., 2012). To our knowledge, use of the more selective NQO1 inhibitor, ES396, has not been reported in chemotherapy sensitivity experiments. However, studies to date suggest that the antioxidant and detoxification properties of NQO1 are likely utilised by PDAC cells to promote proliferation as well as to resist apoptosis and the cytotoxic effects of anti-cancer xenobiotics.

The majority of NQO1 suppression experiments in PDAC have utilised the MiaPaCa-2 cell line. To compare the effects of Nrf2 knockdown to NQO1 suppression, we can consider the findings of Lister et al. who transfected MiaPaCa-2 cells with Nrf2 siRNA. Here, a significant reduction in proliferation on Nrf2 knockdown compared to RNAi control was observed (Lister et al., 2011). As for chemosensitivity, increased apoptosis has been observed with Nrf2 knockdown in MiaPaCa-2 cells treated with the anti-cancer agent etoposide (Arlt et al., 2013). Therefore, both targeted suppression of NQO1, and its upstream regulator, Nrf2, act to promote proliferation and chemoresistance, at least in the MiaPaCa-2 cell line.

3.6.2.2. Aldo-keto reductase family 1 member b10. Another key Nrf2/ARE effector protein that is overexpressed in PDAC, AKR1B10, has important detoxification functions and counters redox cycling through catalysing the reduction of quinones (Hayes and Dinkova-Kostova, 2014). It has a particular role in steroid metabolism and detoxification of aldehydes. AKR1B10 silencing through siRNA knockdown was associated with suppressed cell proliferation and increased apoptosis in the Panc10-05 cell line compared to controls (Chung et al., 2012). Using a different PDAC cell line, CD18, AKR1B10 knockdown inhibited tumour proliferation *in vivo* (Zhang et al., 2014). The contribution to chemoresistance of AKR1B10 has not been investigated in PDAC, but suppressed AKR1B10 activity in colorectal cancer cells has been found to reduce cell viability following treatment from cytotoxic agents (Yan et al., 2007), and it would be likely that overexpression of AKR1B10 contributes to chemoresistance in other cancers. We are not aware of any reports of Nrf2 knockdown using either of these cell lines to drawn comparisons, but from these studies we find that AKR1B10 contributes to PDAC survival and proliferation even in the absence of chemical stress from xenobiotics.

3.6.2.3. Heme oxygenase 1. The gene encoding heme oxygenase 1, *HMOX1*, contains multiple ARE sequences and is highly induced by many chemical and physical cellular stress stimuli, including those relevant to cancer such as oxidative stress, ER stress, UV irradiation, and hypoxia (Ryter et al., 2006). Increased expression levels of HO-1 have been detected in many cancers, and targeted knockdown results in significant inhibition of PDAC cell proliferation (Berberat et al., 2005). Down regulation of HO-1 by siRNA decreased viability of several PDAC cell lines following exposure to radiotherapy or gemcitabine *in vitro* (Berberat et al., 2005). Therefore, upregulation of HO-1 in PDAC also appears to confer proliferative and chemoresistant properties.

3.6.2.4. Thioredoxin reductase 1. Finally, the thioredoxin antioxidant system, which is also under Nrf2/ARE regulation, has an important role in maintaining redox state of thiols in cellular proteins (Arner and Holmgren, 2006). Thioredoxin is overexpressed in human pancreatic cancer specimens and PDAC cell lines (Arnold et al., 2004; Yi and Oh, 2015), and reduced thioredoxin (through thioredoxin reductase) is known to promote proliferation, inhibit apoptosis, and protect cells from oxidative stress (Powis and Montfort, 2001; Arnold et al., 2004; Yan et al., 2012). In PDAC cell lines (i.e. MiaPaca-2, Panc-1 and BxPC-3) treatment with selective thioredoxin reductase 1 (TrxR1) inhibitors induces apoptosis (Yan et al., 2012). To the best of our knowledge, no combination studies with TrxR1 inhibitors and standard chemotherapy agents have been reported using PDAC cell lines. A randomised clinical study of PDAC patients with advanced disease treated with thioredoxin inhibitor monotherapy failed to demonstrate improved outcomes, however considerable methodological issues may have obscured potential benefits of inhibiting this antioxidant system (Ramanathan et al., 2011). Interestingly, TrxR1 may also regulate Nrf2 activation (Cebula et al., 2015).

Taken together, these studies demonstrate that enzymes encoded by ARE-genes such as *NQO1*, *AKR1B10*, *HMOX1* and *TXNRD1* individually provide anti-apoptotic protection of PDAC cell lines, as did Nrf2 knockdown, in the absence of any cytotoxic treatment. As for chemosensitivity, increased apoptosis following Nrf2 knockdown was seen in all PDAC cell lines treated with chemotherapy compounds but drug-specific variability was observed, especially for Suit-2 and FAMPAC cells (Lister et al., 2011). Whilst these cell lines both have activating mutant *K-Ras*, there are differences in basal Nrf2 and Keap1 expression that will likely alter the effects of Nrf2 knockdown (Lister et al., 2011; Naumann et al., 1996; Moore et al., 2001). There is limited information on chemosensitivity of PDAC cells from selected inhibition of enzymes from ARE-driven genes, but it has been demonstrated that targeted knockdown of *NQO1* and *HO-1* result in increased chemosensitivity (Ough et al., 2005; Berberat et al., 2005). Unlike the selected the Nrf2/ARE effector proteins discussed, Nrf2 is also able to modulate other important protective systems relevant to chemoresistance such as proteasomal gene expression and multi-drug-resistance associated protein transporters (MRPs), providing additional cytoprotective functions compared with increased levels of individual drug detoxification and antioxidant proteins (Duong et al., 2014; Hong et al., 2010; Mohelnikova-Duchonova et al., 2013).

3.7. Nrf2 inhibitor therapy for pancreatic cancer

3.7.1. Nrf2 inhibitors

In view of the role of Nrf2 in pancreatic carcinogenesis and chemoresistance, a strategy for selectively inhibiting Nrf2 would appear to have potential therapeutic promise. Indeed, this is a proposed approach for a variety of other common cancers (e.g. lung, colon, head and neck, breast, and endometrial) as well as some non-neoplastic diseases (Coppole, 2012). To date, there is a small but growing number of Nrf2 inhibitor compounds, all of which are naturally occurring, namely; retinoic acid (Wang et al., 2007), brusatol (Ren et al., 2011; Olayanju et al., 2015), luteolin (Tang et al., 2011), and trigonelline (Boettler et al., 2011). The latter two have been studied in pancreatic cancer.

From an investigation of a panel of flavonoids, luteolin (3',4',5',7-tetrahydroxyflavone) was uniquely identified as a potent Nrf2 inhibitor, through its ability to enhance Nrf2 mRNA decay (Tang et al., 2011). As such, luteolin suppressed Nrf2-target gene expression in a dose-dependant manner (Tang et al., 2011). Additionally, luteolin is recognised for its ability to inhibit PDAC cell line proliferation (Cia et al., 2012; Johnson and Gonzalez de Mejia, 2013)

and recently was shown to augment the apoptotic effect of gemcitabine *in vivo* when administered in combination (Johnson et al., 2015). It is important to note, however, that luteolin is not a specific Nrf2 inhibitor, and the anti-cancer effects seen in PDAC are likely related to the impact luteolin has on several signalling pathways. Besides Nrf2 regulation, luteolin can dramatically alter cell cycle control and induce apoptosis through cyclooxygenase-2, NFκB, and Bcl-xL (*B-cell lymphoma-extra large*) due to its core flavone chemical structure (Wenzel et al., 2000). Luteolin has also been shown to specifically inhibit other key pathways involved in angiogenesis, proliferation and inflammation through reduced vascular endothelial growth factor (Bagli et al., 2004; Cai et al., 2012), inhibited JNK (*c-Jun N-terminal kinase*) and AP-1 (*activator protein 1*) signalling (Jang et al., 2008), STAT3 degradation (Selvendiran et al., 2006), and p53 stabilisation (López-Lázaro, 2009). The studies of luteolin presented to date in PDAC cell lines did not specifically determined whether the pro-apoptotic effects of luteolin were dependent upon Nrf2 inhibition (Cai et al., 2012; Johnson and Gonzalez de Mejia, 2013; Johnson et al., 2015).

Trigonelline (1-methylpyridinium-3-carboxylate), a major alkaloid coffee bean extract, was first reported as an inhibitor of Nrf2/ARE dependent gene expression in colon cancer cells (Boettler et al., 2011). More recently, trigonelline has been tested in PDAC cell lines where it was found to inhibit the ability of Nrf2 to translocate into the nucleus (Arlt et al., 2013). Importantly, the sensitivity of PDAC cells to anti-cancer drugs was increased with trigonelline treatment (Arlt et al., 2013). In this study, the suppression of proteasomal activity by trigonelline in PDAC cell lines was clearly demonstrated to be dependent upon Nrf2 by using a targeted knockdown approach (Arlt et al., 2013). The greatest inhibitory effects of trigonelline upon Nrf2/ARE signalling in both pancreatic and colon cancer cells were at submaximal doses, with higher levels being less efficient or ineffective (Arlt et al., 2013; Boettler et al., 2011).

In non-PDAC cancer studies, brusatol has been shown to be a highly specific Nrf2 inhibitor which produces rapid and transient effects, and can increase chemotherapy sensitisation (Olayanju et al., 2015; Ren et al., 2011). Thus brusatol would be a useful experimental tool for further study of Nrf2 in PDAC, although the precise mechanism of Nrf2 inhibition is still under investigation. The Nrf2/ARE suppressive effects of retinoic acid have been shown to be mediated through direct binding to Nrf2 by retinoic X receptor alpha in the nucleus, a mechanism which augments cancer cell chemosensitivity and may also be useful for studying Nrf2 inhibition in PDAC (Wang et al., 2007; Valenzuela et al., 2014).

Although complicated by dosing considerations, Nrf2 inhibitors provide useful tools in exploring the mechanisms and effects of Nrf2 inhibition in the preclinical setting, from which assessments can be made as to the therapeutic context that these might be safely be applied to PDAC and other diseases with Keap1–Nrf2 dysregulation (Chapple et al., 2012; Coppole, 2012; Olayanju et al., 2015).

3.7.2. Challenges in the application of Nrf2 inhibitor therapy for pancreatic cancer

The study of Nrf2 inhibitors in pancreatic cancer models prompts consideration of how Nrf2 targeted therapies could be safely and effectively applied to PDAC patients. Notwithstanding the issues of patient selection and of delivering relevant drug concentrations to the cancer tissues, there remain two particular difficulties which may preclude the use of targeted Nrf2 inhibitors for PDAC which relate to the suppression of Nrf2 in non-cancerous host tissue. Firstly, there may be an intolerable or unacceptable side-effect profile when using anti-cancer cytotoxic drugs in combination with Nrf2 inhibitor therapy, as a result

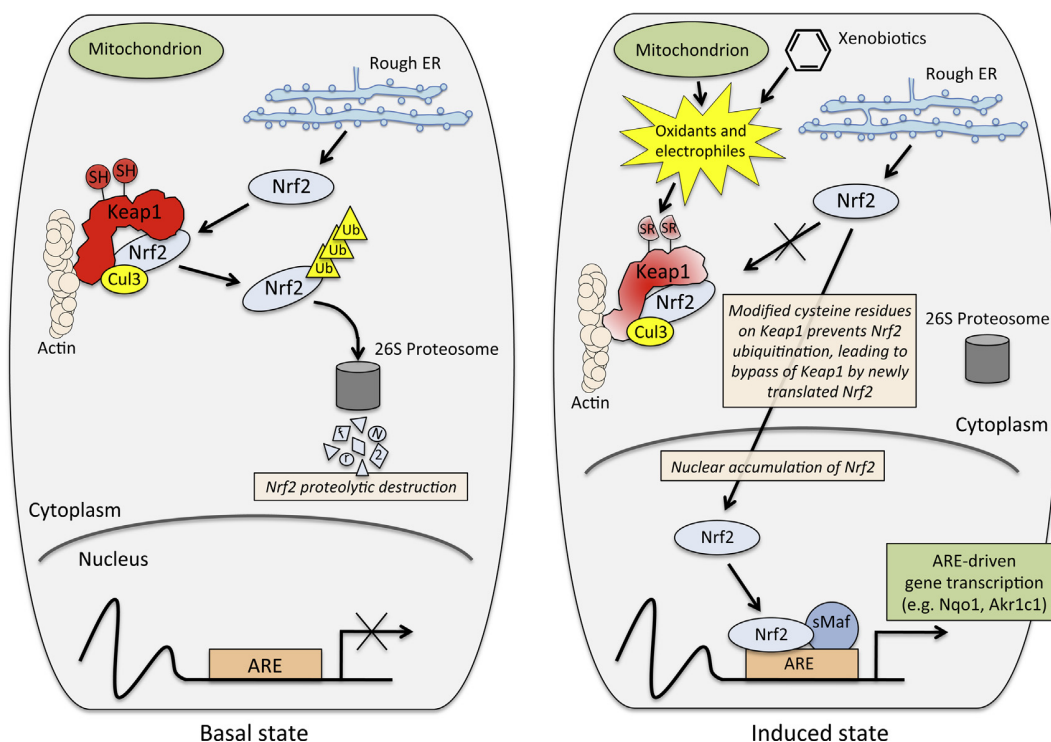


Fig. A1. Overview of the Keap1–Nrf2–ARE system. The schematic illustrates that under normal homeostatic conditions (basal state), Nrf2 is prevented from associating with the antioxidant response element (ARE) since the Nrf2 protein is targeted by Cullin-3 (Cul3) for proteasomal degradation by Keap1. Under conditions of cellular stress (induced state), cysteine residues on Keap1 are modified by oxidants and electrophiles, impeding the binding of Nrf2 which is free to translocate to the nucleus. Inside the nucleus, Nrf2 forms a heterodimer with small Maf protein (sMaf) and activates transcription of selected genes, such as *NQO1* and *AKR1C1*, which contain the ARE gene promoter region.

suppressed cytoprotective systems in non-target cells. Secondly, there is at present little known of the effects that modulating Nrf2–ARE signalling might have upon interactions between tumour metastasis and the host, which may alter the biological behaviour of the metastasis. Indeed, there is evidence that Nrf2 may be protective against cancer metastasis (Satoh et al., 2010). Further preclinical investigation into the mechanistic role of Nrf2 in PDAC metastasis would clearly be important.

4. Conclusions

Nrf2 is frequently upregulated in PDAC and is at least in part, likely to be oncogene driven, and contributes to proliferation and chemoresistance. Raised Nrf2 levels in PDAC occur in the absence of somatic gene mutations in *NFE2L2* or its dominant negative regulator, *KEAP1*. Of prognostic importance, increased tumour nuclear Nrf2 expression correlates with reduced survival. Targeted Nrf2 inhibitors have been found to be therapeutically beneficial when administered in combination with conventional anti-cancer drugs in preclinical PDAC studies by increasing chemosensitivity. The introduction of Nrf2 inhibitors into the clinical setting for pancreatic cancer is a promising avenue but presents some challenges.

Authors' contribution

AJH and CS wrote the article. BH and RMC reviewed the manuscript.

Acknowledgements

We are most grateful to Professor John D. Hayes (University of Dundee), Dr. Fiona Oakley (Newcastle University) and Mr. Damian J. Mole (University of Edinburgh) for their valuable discussions and advice in preparing the manuscript.

Appendix A.

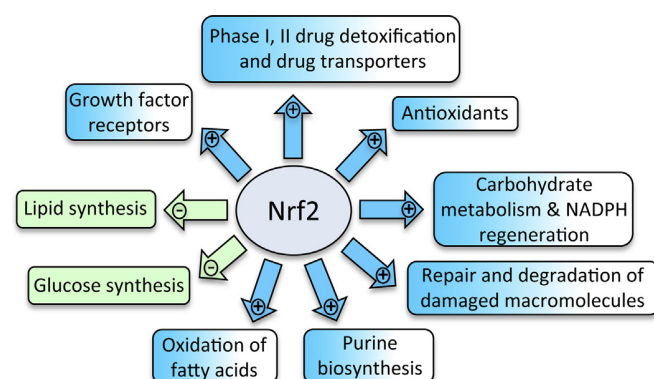


Fig. A2. Downstream functions of the Nrf2–ARE pathway. Nrf2 inhibits lipid and glucose synthesis, and activates several cytoprotective mechanisms which serve to prevent or control cellular damage and activate repair and regeneration.

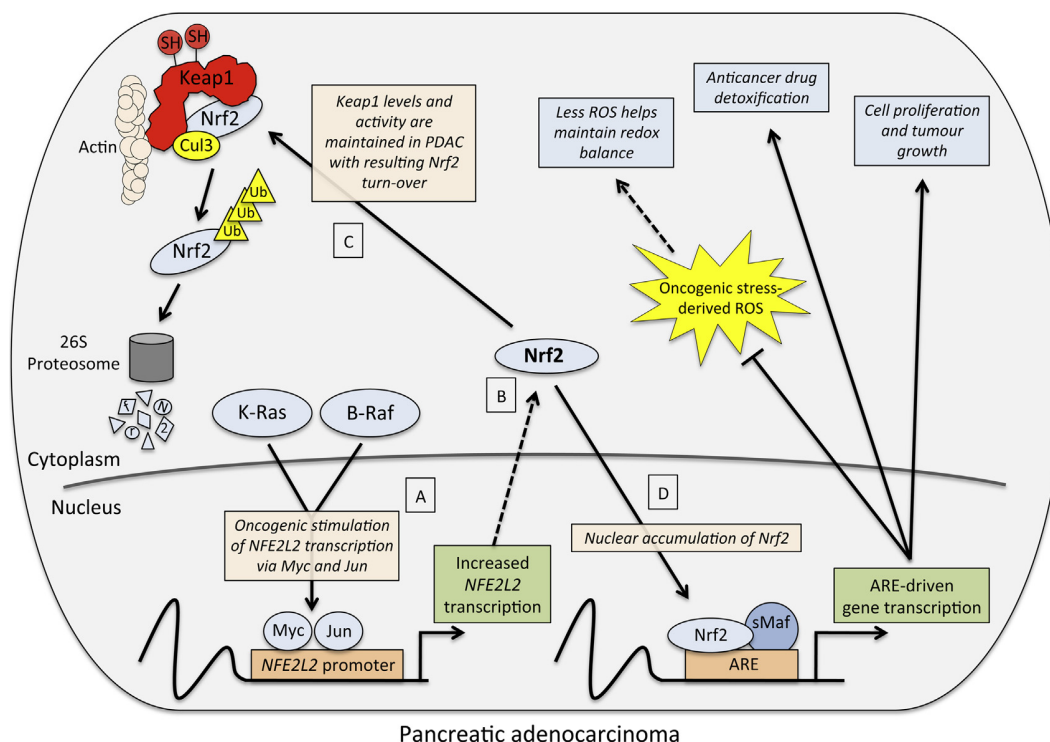


Fig. A3. Keap1–Nrf2 signalling in pancreatic cancer. The schematic illustrates oncogene-driven *NFE2L2* transcription in pancreatic cancer (A), resulting in high levels of Nrf2 protein being translated (B). Whilst a proportion of Nrf2 protein will continue to be degraded via Keap1-mediated ubiquitination by Cul3 (C), the much increased *NFE2L2* transcription results in greater availability of Nrf2 protein to translocate into the nucleus (D), which results in heterodimerization with small Maf protein and upregulation of ARE-driven gene transcription. In this way, cancer cells can gain several selective advantages including increased proliferation, enhanced drug detoxification, and improved redox homeostasis by increased scavenging of reactive oxygen species (ROS).

Table A1

Basal protein expression levels of Nrf2, Keap1, and downstream Nrf2/ARE effector proteins (NQO1, AKR1B10, AKR1C1) across commonly used pancreatic cancer cell lines compared with mutant *KRAS* status.

Cell line	Nrf2 protein	Keap1 protein	Nrf2/ARE effector proteins			Activating mutation
			NQO1	AKR1B10	AKR1C1	
AsPC-1	High (Hong et al., 2010)	–	High (Hong et al., 2010; Cullen et al., 2003; Lewis et al., 2004)	–	–	Mutant (Moore et al., 2001; Loukopoulos et al., 2004)
BxPC-3	–	–	High (Cullen et al., 2003; Dehn et al., 2006; Lewis et al., 2004)	–	–	WT (Loukopoulos et al., 2004; Naumann et al., 1996; Berrozpe et al., 1994)
Capan-1	High (Hong et al., 2010)	–	High (Cullen et al., 2003)	High (Chung et al., 2012)	–	Mutant (Loukopoulos et al., 2004; Naumann et al., 1996; Berrozpe et al., 1994)
Colo-357	High (Hong et al., 2010; Arlt et al., 2013)	–	High (Hong et al., 2010)	–	–	Mutant (Naumann et al., 1996)
FAMPAC	Undetected (Lister et al., 2011)	High (Lister et al., 2011)	–	–	Low (Lister et al., 2011)	Mutant (Fredebohm et al., 2012)
MiaPaca-2	Undetected (Lister et al., 2011). Detected, no increase (Arlt et al., 2013)	High (Lister et al., 2011)	High (Cullen et al., 2003; Dehn et al., 2006; Lewis et al., 2004)	–	High (Lister et al., 2011)	Mutant (Moore et al., 2001)
Panc-1	Undetected (Lister et al., 2011). High (Hong et al., 2010; Arlt et al., 2013)	High (Lister et al., 2011)	Undetected (Hong et al., 2010)	–	High (Lister et al., 2011)	Mutant (Moore et al., 2001)
Suit-2	High (Lister et al., 2011)	Low (Lister et al., 2011)	–	–	High (Lister et al., 2011)	Mutant (Moore et al., 2001)

The protein levels reported in the table reflect expression compared with controls by immunoblot. 'WT' is wild-type. Variability is found across pancreatic cancer cell lines in basal Nrf2, Keap1 and Nrf2/ARE effector protein levels. There appears to be no clear association between mutant *KRAS* status and expression of Nrf2 and ARE-target genes, which suggests that additional factors outwith the Keap1–Nrf2 pathway contribute significantly.

References

- Acharya, A., Das, I., Chandhok, D., Saha, T., 2010. Redox regulation in cancer: a double-edged sword with therapeutic potential. *Oxid. Med. Cell. Longev.* 3 (1), 23–34.
- Agyeman, A.S., Chaekady, R., Shaw, P.G., Davidson, N.E., Visvanathan, K., Pandey, A., Kensler, T.W., 2012. Transcriptional and proteomic profiling of KEAP1 disrupted and sulphoraphane-treated human breast epithelial cells reveals common expression profiles. *Breast Cancer Res. Treat.* 132 (1), 175–187.
- Al Saati, T., Clerc, P., Hanoun, N., Peugeot, S., Lulka, H., Gigoux, V., Capilla, F., Béluchon, B., Couvelard, A., Selves, J., Buscall, L., Carrier, A., Dusetti, N., Dufresne, M., 2013. Oxidative stress induced by inactivation of TP53INP1 cooperates with KrasG12D to initiate and promote pancreatic carcinogenesis in the murine pancreas. *Am. J. Pathol.* 182 (6), 1996–2004.
- Altomare, E., Grattagliano, I., Vendemiale, G., Palmieri, V., Palasciano, G., 1996. Acute ethanol administration induces oxidative changes in rat pancreatic tissue. *Gut* 38 (5), 742–745.
- Arlt, A., Bauer, I., Schafmayer, C., Tepel, J., Mürköster, S.S., Brosch, M., Röder, C., Kalthoff, H., Hampe, J., Moyer, M.P., Fölsch, U.R., Schäfer, H., 2009. Increased proteasome subunit protein expression and proteasome activity in colon cancer relate to an enhanced activation of nuclear factor E2-related factor 2 (Nrf2). *Oncogene* 28 (45), 3983–3996.
- Arlt, A., Sebens, S., Krebs, S., Geismann, C., Grossmann, M., Kruse, M.L., Schreiber, S., Schäfer, H., 2013. Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. *Oncogene* 32 (40), 4825–4835.
- Arner, E.S., Holmgren, A., 2006. The thioredoxin system in cancer. *Semin. Cancer Biol.* 16 (6), 420–426.
- Arnold, N.B., Ketterer, K., Kleeff, J., Friess, H., Buchler, M.W., Korc, M., 2004. Thioredoxin is downstream of Smad7 in a pathway that promotes growth and suppresses cisplatin-induced apoptosis in pancreatic cancer. *Cancer Res.* 64 (10), 3599–3606.
- Awadallah, N.S., Dehn, D., Shah, R.J., Russell Nash, S., Chen, Y.K., Ross, D., Bentz, J.S., Shroyer, K.R., 2008. NQO1 expression in pancreatic cancer and its potential use as a biomarker. *Appl. Immunohistochem. Mol. Morphol.* 16 (1), 24–31.
- Bagli, E., Stefanitou, M., Morbidelli, L., Ziche, M., Psillas, K., Murphy, C., Fotsis, T., 2004. Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity. *Cancer Res.* 64 (21), 7936–7946.
- Bai, H., Li, H., Zhang, W., Matkowskyj, K.A., Liao, J., Srivastava, S.K., Yang, G.Y., 2011. Inhibition of chronic pancreatitis and pancreatic intraepithelial neoplasia (PanIN) by capsaicin in LSL-KrasG12D/Pdx1-Cre mice. *Carcinogenesis* 32 (11), 1689–1696.
- Berberat, P.O., Dambrasas, Z., Gulbinas, A., Giese, T., Giese, N., Künzli, B., Autschbach, F., Meuer, S., Büchler, M.W., Friess, H., 2005. Inhibition of heme oxygenase-1 increases responsiveness of pancreatic cancer cells to anti-cancer treatment. *Clin. Cancer Res.* 11 (10), 3790–3798.
- Baird, L., Lleres, D., Swift, S., Dinkova-Kostova, A.T., 2013. Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1–Nrf2 protein complex. *Proc. Natl. Acad. Sci. U. S. A.* 110 (38), 15259–15264.
- Berrozpe, G., Schaeffer, J., Peinado, M.A., Real, F.X., Perucho, M., 1994. Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. *Int. J. Cancer* 58 (2), 185–191.
- Boettler, U., Sommerfeld, K., Volz, N., Pahlke, G., Teller, N., Somoza, V., Lang, R., Hofmann, T., Marko, D., 2011. Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (Epre)-dependent gene expression. *J. Nutr. Biochem.* 22 (5), 424–440.
- Cano, C.E., Gommeaux, J., Pietri, S., Culcasi, M., Garcia, S., Seux, M., Barelire, S., Vasseur, S., Spoto, R.P., Pébusque, L.J., Dusetti, N.J., Iovanna, J.L., Carrier, A., 2009. Tumor protein 53-induced nuclear protein 1 is a major mediator of p53 antioxidant function. *Cancer Res.* 69 (1), 219–226.
- Cai, X., Lu, W., Ye, T., Lu, M., Wang, J., Huo, J., Qian, S., Wang, X., Cao, P., 2012. The molecular mechanism of luteolin-induced apoptosis is potentially related to inhibition of angiogenesis in human pancreatic carcinoma cells. *Oncol. Rep.* 28 (4), 1353–1361.
- Cebula, M., Schmidt, E.E., Arner, E.S., 2015. TrxR1 as a potent regulator of the Nrf2–Keap1 response system. *Antioxid. Redox Signal.* (Epub ahead of print).
- Chang, D.K., Grimmond, S.M., Biankin, A.V., 2014. Pancreatic cancer genomics. *Curr. Opin. Genet. Dev.* 24, 74–81.
- Chapple, S.J., Siow, R.C.M., Mann, G.E., 2012. Crosstalk between Nrf2 and the proteasome: therapeutic potential of Nrf2 inducers in vascular disease and aging. *Int. J. Biochem. Cell Biol.* 44 (8), 1315–1320.
- Chen, N., Yi, X., Abushahin, N., Pang, S., Zhang, D., Kong, B., Zheng, W., 2010. Nrf2 expression in endometrial serous carcinomas and its precancers. *Int. J. Clin. Exp. Pathol.* 4 (1), 85–96.
- Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A., Hayes, J.D., 2013. Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 32 (32), 3765–3781.
- Chung, Y.T., Matkowskyj, K.A., Li, H., Bai, H., Zhang, W., Tsao, M.S., Liao, J., Yang, G.Y., 2012. Overexpression and oncogenic function of aldo–keto reductase family 1B10 (AKR1B10) in pancreatic carcinoma. *Mod. Pathol.* 25 (5), 758–766.
- Cia, X., Lu, W., Ye, T., Lu, M., Wang, J., Huo, J., Qian, S., Wang, X., Cao, P., 2012. The molecular mechanism of luteolin-induced apoptosis is potentially related to inhibition of angiogenesis in human pancreatic carcinoma cells. *Oncol. Rep.* 28 (4), 1353–1361.
- Copple, I.M., 2012. The Keap1–Nrf2 cell defence pathway – a promising therapeutic target? *Adv. Pharmacol.* 63, 43–79.
- Cullen, J.J., Hinkhouse, M.M., Grady, M., Gaut, A.W., Liu, J., Zhang, Y.P., Weydert, C.J., Domann, F.E., Oberley, L.W., 2003. Dicumarol inhibition of NADPH:quinone oxidoreductase induces growth inhibition of pancreatic cancer via a superoxide-mediated mechanism. *Cancer Res.* 63 (17), 5513–5520.
- Cullinan, S.B., Diehl, J.A., 2004. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum. *J. Biol. Chem.* 279 (19), 20108–20117.
- Cullinan, S.B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R.J., Diehl, J.A., 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol.* 23 (20), 7198–7209.
- Dehn, D.L., Siegel, D., Zafar, K.S., Reigan, P., Swann, E., Moody, C.J., Ross, D., 2006. 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxymethyl)indole-4,7-dione, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, exhibits activity against human pancreatic cancer in vitro and in vivo. *Mol. Cancer Ther.* 5 (7), 1702–1709.
- DeNicola, G.M., Karreth, F.A., Humpton, T.J., Gopinathan, A., Wei, C., Frese, K., Mangal, D., Yu, K.H., Yeo, C.J., Calhoun, E.S., Scrimieri, F., Winter, J.M., Hruban, R.H., Iacobuzio-Donahue, C., Kern, S.E., Blair, I.A., Tuveson, D.A., 2011. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475 (7354), 106–109.
- Devling, T.W., Lindsay, C.D., McLellan, L.I., McMahon, M., Hayes, J.D., 2005. Utility of siRNA against Keap1 as a strategy to stimulate a cancer chemopreventive phenotype. *Proc. Natl. Acad. Sci. U. S. A.* 102 (20), 7280–7285.
- Dunn, L.L., Midwinter, R.G., Ni, J., Hamid, H.A., Parish, C.R., Stoker, R., 2014. New insights into intracellular locations and functions of heme oxygenase-1. *Antioxid. Redox Signal.* 20 (11), 1723–1742.
- Duong, H.Q., Yi, Y.W., Kang, H.J., Hong, Y.B., Tang, W., Wang, A., Seong, Y.S., Bae, I., 2014. Inhibition of Nrf2 by PIK-75 augments sensitivity of pancreatic cancer cells to gemcitabine. *Int. J. Oncol.* 44 (3), 959–969.
- Eades, G., Yang, M., Yao, Y., Zhang, Y., Zhou, Q., 2011. miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *J. Biol. Chem.* 286 (47), 40725–40733.
- Fourquet, S., Guerois, R., Biard, D., Toledano, M.B., 2010. Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation. *J. Biol. Chem.* 285 (11), 8463–8471.
- Fredebohm, J., Boettcher, M., Eisen, C., Gaida, M.M., Heller, A., Keleg, S., Tost, J., Greulich-Bode, K.M., Hotz-Wagenblatt, A., Lathrop, M., Giese, N.A., Hoheisel, J.D., 2012. Establishment and characterization of a highly tumorigenic and cancer stem cell enriched pancreatic cancer cell line as a well defined model system. *PLoS ONE* 7 (11), e48503.
- Ganesh, P.C., Sreejayan, Rao, M.N., 1999. Evidence for oxidant stress in chronic pancreatitis. *Indian J. Gastroenterol.* 18 (4), 156–157.
- Gironella, M., Seux, M., Xie, M.J., Cano, C., Tomasini, R., Gommeaux, J., Garcia, S., Nowak, J., Yeung, M.L., Jeang, K.T., Chaix, A., Fazli, L., Motoo, Y., Wang, Q., Rocchi, P., Russo, A., Gleave, M., Dagorn, J.C., Iovanna, J.L., Carrier, A., Pébusque, M.J., Dusetti, N.J., 2007. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155 and its restoration inhibits tumour development. *Proc. Natl. Acad. Sci. U. S. A.* 104 (41), 16170–16175.
- Guerra, C., Schuhmacher, A.J., Cañamero, M., Grippo, P.J., Verdaguier, L., Pérez-Gallego, L., Dubus, P., Sandgren, E.P., Barbacid, M., 2007. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* 11 (3), 291–302.
- Halliwell, B., Gutteridge, J.M.C., 2007. *Free Radicals in Biology and Medicine*, 4th ed. Oxford University Press.
- Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell* 144 (5), 646–674.
- Hayes, J.D., Chanas, S.A., Henderson, C.J., McMahon, M., Sun, C., Moffat, G.J., Wolf, C.R., Yamamoto, M., 2000. The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem. Soc. Trans.* 28 (2), 33–41.
- Hayes, J.D., Kelleher, M.O., Eggleston, I.M., 2008. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *Eur. J. Nutr.* 47 (Suppl. 2), 73–88.
- Hayes, J.D., McMahon, M., 2009. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends Biochem. Sci.* 34 (4), 176–188.
- Hayes, J.D., Dinkova-Kostova, A.T., 2014. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* 39 (4), 199–218.
- Higgins, L.G., Kelleher, M.O., Eggleston, I.M., Itoh, K., Yamamoto, M., Hayes, J.D., 2009. Transcription factor Nrf2 mediates an adaptive response to sulforaphane that protects fibroblasts in vitro against the cytotoxic effects of electrophiles, peroxides and redox-cycling agents. *Toxicol. Appl. Pharmacol.* 237 (3), 267–280.
- Homma, S., Ishii, Y., Morishima, Y., Yamadori, T., Matsuno, Y., Haraguchi, N., Kikuchi, N., Satoh, H., Sakamoto, T., Hizawa, N., Itoh, K., Yamamoto, M., 2009. Nrf2 enhances cell proliferation and resistance to anti-cancer drugs in human lung cancer. *Clin. Cancer Res.* 15 (10), 3423–3432.

- Hong, Y.B., Kang, H.J., Kwon, S.Y., Kim, H.J., Kwon, K.Y., Cho, C.H., Lee, J.M., Kallakury, B.V., Bae, I., 2010. Nuclear factor (erythroid-derived 2)-like 2 regulates drug resistance in pancreatic cancer cells. *Pancreas* 39 (4), 463–472.
- Hourihan, J.M., Kenna, J.G., Hayes, J.D., 2013. The gasotransmitter hydrogen sulfide induces Nrf2-target genes by inactivating the Keap1 ubiquitin ligase substrate adaptor through formation of a disulfide bond between cys-226 and cys-613. *Antioxid. Redox Signal.* 19 (5), 465–481.
- Ino, Y., Yamazaki-Itoh, R., Shimada, K., Iwasaki, M., Kosuge, T., Kanai, Y., Hiraoka, N., 2013. Immune cell infiltrate as an indicator of the immune microenvironment of pancreatic cancer. *Br. J. Cancer* 108 (4), 914–923.
- Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* 236 (2), 313–322.
- Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., Yamamoto, M., 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13 (1), 76–86.
- Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., Yamamoto, M., 2003. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 8 (4), 379–391.
- Jang, S., Kelly, K., Johnson, R.W., 2008. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. *Proc. Natl. Acad. Sci. U. S. A.* 105 (21), 7534–7539.
- Johnson, J.L., Gonzalez de Mejia, E., 2013. Interactions between dietary flavonoids apigenin or luteolin and chemotherapeutic drugs to potentiate anti-proliferative effect on human pancreatic cancer cells, in vitro. *Food Chem. Toxicol.* 60, 83–91.
- Johnson, J.L., Dia, V.P., Wallig, M., Gonzalez de Mejia, E., 2015. Luteolin and gemcitabine protect against pancreatic cancer in an orthotopic mouse model. *Pancreas* 44 (1), 144–151.
- Jones, S., Zhang, X., Parsons, D.W., et al., 2008. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* 321 (5897), 1801–1806.
- Ju, H.Q., Gocho, T., Aguilar, M., Wu, M., Zhuang, Z.N., Fu, J., Yanaga, K., Huang, P., Chiao, P.J., 2015. Mechanisms of overcoming intrinsic resistance to in pancreatic ductal adenocarcinoma through the redox modulation. *Mol. Cancer Ther.* 14 (3), 788–798.
- Kim, Y., Han, D., Min, H., Jin, J., Yi, E.C., Kim, Y., 2014. Comparative proteomic profiling of pancreatic ductal adenocarcinoma cell lines. *Mol. Cells* 37 (12), 888–898.
- Kong, B., Qia, C., Erkan, M., Kleeff, J., Michalski, C.W., 2013. Overview on how oncogenic Kras promotes pancreatic carcinogenesis by inducing low intracellular ROS levels. *Front. Physiol.* 12 (4), 246.
- Kwak, M.K., Wakabayashi, N., Greenlaw, J.L., Yamamoto, M., Kensler, T.W., 2003. Antioxidants enhance mammalian proteasome expression through Keap1-Nrf2 signaling pathway. *Mol. Cell. Biol.* 23 (23), 8786–8794.
- Lau, A., Villeneuve, N.F., Sun, Z., Wong, P.K., Zhang, D.D., 2008. Dual roles of Nrf2 in cancer. *Pharmacol. Res.* 58 (5–6), 262–270.
- Lesina, M., Kurkowski, M.U., Ludes, K., Rose-John, S., Treiber, M., Klöppel, G., Yoshimura, A., Reindl, W., Sipos, B., Akira, S., Schmid, R.M., Algül, H., 2011. Stat3/Socs3 activation by IL-6 transsignalling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell* 19 (4), 456–469.
- Lewis, A., Ough, M., Li, L., Hinkhouse, M.M., Ritchie, J.M., Spitz, D.R., Cullen, J.J., 2004. Treatment of pancreatic cancer cells with dicumarol induces cytotoxicity and oxidative stress. *Clin. Cancer Res.* 10 (13), 4550–4558.
- Lewis, A.M., Ough, M., Hinkhouse, M.M., Tsao, M.S., Oberley, L.W., Cullen, J.J., 2005. Targeting NAD(P)H:quinone oxidoreductase (NQO1) in pancreatic cancer. *Mol. Carcinog.* 43 (4), 215–224.
- Li, H., Yang, A.L., Chung, Y.T., Zhang, W., Liao, J., Yang, G.Y., 2013. Sulindac inhibits pancreatic carcinogenesis in LSL-KrasG12D-LSL-Trp53R172H-Pdx-1-Cre mice via suppressing aldo-keto reductase family 1B10 (AKR1B10). *Carcinogenesis* 34 (9), 2090–2098.
- Li, L.S., Bey, E.A., Dong, Y., Meng, J., Patra, B., Yan, J., Xie, X.J., Brekken, R.A., Barnett, C.C., Bornmann, W.G., Gao, J., Boothman, D.A., 2011. Modulating endogenous NQO1 levels identifies key regulatory mechanisms of action of β -lapachone for pancreatic cancer therapy. *Clin. Cancer Res.* 17 (2), 275–285.
- Lister, A., Nedjadi, T., Kitteringham, N.R., Campbell, F., Costello, E., Lloyd, B., Copple, I.M., Williams, S., Owen, A., Neoptolemos, J.P., Goldring, C.E., Park, B.K., 2011. Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy. *Mol. Cancer* 10, 37.
- Logsdon, C.D., Simeone, D.M., Binkley, C., Arumugam, T., Greenson, J.K., Giordano, T.J., Misk, D.E., Kuick, R., Hanash, S., 2003. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Res.* 63 (10), 2649–2657.
- Logsdon, C.D., Ji, B., 2013. The role of protein synthesis and digestive enzymes in acinar cell injury. *Nat. Rev. Gastroenterol. Hepatol.* 10 (6), 362–370.
- López-Lázaro, M., 2009. Distribution and biological activities of the flavonoid luteolin. *Mini Rev. Med. Chem.* 9 (1), 31–59.
- Loukopoulou, P., Kanetaka, K., Takamura, M., Shibata, T., Sakamoto, M., Hirohashi, S., 2004. Orthotopic transplantation models of pancreatic adenocarcinoma derived from cell lines and primary tumors and displaying varying metastatic activity. *Pancreas* 29 (3), 193–203.
- Lu, J., Holmgren, A., 2014. The thioredoxin antioxidant system. *Free Radic. Biol. Med.* 66, 75–87.
- Lyn-Cook, B.D., Yan-Sanders, Y., Moore, S., Taylor, S., Word, B., Hammons, G.J., 2006. Increased levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) in pancreatic tissues from smokers and pancreatic adenocarcinomas: a potential biomarker of early damage in the pancreas. *Cell Biol. Toxicol.* 22 (2), 73–80.
- McMahon, M., Itoh, K., Yamamoto, M., Hayes, J.D., 2003. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J. Biol. Chem.* 278 (24), 21592–21600.
- McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., Hayes, J.D., 2006. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a “tethering” mechanism: a two-site interaction model for the Nrf2–Keap1 complex. *J. Biol. Chem.* 281 (34), 24756–24768.
- McWalter, G.K., Higgins, L.G., McLellan, L.L., Henderson, C.J., Song, L., Thornalley, P.J., Itoh, K., Yamamoto, M., Hayes, J.D., 2004. Transcription factor Nrf2 is essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates. *J. Nutr.* 134 (Suppl. 12), 3499S–3506S.
- Meakin, P.J., Chowdhry, S., Sharma, R.S., Ashford, F.B., Walsh, S.V., McCrimmon, R.J., Dinkova-Kostova, A.T., Dillon, J.F., Hayes, J.D., Ashford, M.L., 2014. Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Mol. Cell. Biol.* 34 (17), 3305–3320.
- Mitsuishi, Y., Taguchi, K., Kawatani, Y., Shibata, T., Nukiwa, T., Aburatani, H., Yamamoto, M., Motohashi, H., 2012. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* 22 (1), 66–79.
- Mohelnikova-Duchonova, B., Brynychova, V., Oliverius, M., Honsova, E., Kala, Z., Muckova, K., Soucek, P., 2013. Differences in transcript levels of ABC transporters between pancreatic adenocarcinoma and nonmalignant tissues. *Pancreas* 42 (4), 707–716.
- Moore, P.S., Sipos, B., Orlandini, S., Sorio, C., Real, F.X., Lemoine, N.R., Gress, T., Bassi, C., Klöppel, G., Kalthoff, H., Ungefroren, H., Löhr, M., Scarpa, A., 2001. Genetic profile of 22 pancreatic carcinoma cell lines. Analysis of K-ras, p53, p16 and DPC4/Smad4. *Virchows Arch.* 439 (6), 798–802.
- Motohashi, H., Yamamoto, M., 2004. Nrf2–Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* 10 (11), 549–557.
- Muscarella, L.A., Parrella, P., D'Alessandro, V., la Torre, A., Barbano, R., Fontana, A., Tancredi, A., Guarnieri, V., Balsamo, T., Coco, M., Copetti, M., Pellegrini, F., De Bonis, P., Bisceglia, M., Scaramuzzi, G., Maiello, E., Valori, V.M., Merla, G., Vendemiale, G., Fazio, V.M., 2011. Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer. *Epigenetics* 6 (6), 710–719.
- Naumann, M., Savitskaia, N., Eilert, C., Schramm, A., Kalthoff, H., Schmiegel, W., 1996. Frequent codeletion of p16/MTS1 and p15/MTS2 and genetic alterations in p16/MTS1 in pancreatic tumors. *Gastroenterology* 110 (4), 1215–1224.
- Nioi, P., Hayes, J.D., 2004. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. *Mutat. Res.* 555 (1–2), v149–v171.
- Nolan, K.A., Zhao, H., Faulder, P.F., Frenkel, A.D., Timson, D.J., Siegel, D., Ross, D., Burke Jr., T.R., Stratford, I.J., Bryce, R.A., 2007. Coumarin-based inhibitors of human NAD(P)H:quinone oxidoreductase-1. Identification, structure-activity, off-target effects and in vitro human pancreatic cancer toxicity. *J. Med. Chem.* 50 (25), 6316–6325.
- O'Byrne, K.J., Dalglish, A.G., 2001. Chronic immune activation and inflammation as the cause of malignancy. *Br. J. Cancer* 85 (4), 473–483.
- Olayanju, A., Copple, I.M., Bryan, H.K., Edge, G.T., Sison, R.L., Wong, M.W., Lai, Z.Q., Lin, Z.X., Dunn, K., Sanderson, C.M., Alghanem, A.F., Cross, M.J., Ellis, E.C., Ingelman-Sundberg, M., Malik, H.Z., Kitteringham, N.R., Goldring, C.E., Park, B.K., 2015. Brusatol provokes a rapid and transient inhibition of Nrf2 signalling and sensitizes mammalian cells to chemical toxicity – implications for therapeutic targeting of Nrf2. *Free Radic. Biol. Med.* 78, 202–212.
- Ough, M., Lewis, A., Bey, E.A., Gao, J., Ritchie, J.M., Bornmann, W., Boothman, D.A., Oberley, L.W., Cullen, J.J., 2005. Efficacy of beta-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. *Cancer Biol. Ther.* 4 (1), 95–102.
- Padmanabhan, B., Tong, K.I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsui, M., Kang, M.I., Kobayashi, A., Yokoyama, S., Yamamoto, M., 2006. *Mol. Cell* 21 (5), 689–700.
- Powis, G., Montfort, W.R., 2001. Properties and biological activities of thioredoxins. *Annu. Rev. Pharmacol. Toxicol.* 41, 261–295.
- Rada, P., Rojo, A.I., Chowdhry, S., McMahon, M., Hayes, J.D., Cuadrado, A., 2011. SCF/ β -TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol. Cell. Biol.* 31 (6), 1121–1133.
- Ramanathan, R.K., Abbruzzese, J., Dragovich, T., Kirkpatrick, L., Guillen, J.M., Baker, A.F., Pestano, L.A., Green, S., Von Hoff, D.D., 2011. A randomized phase II study of PX-12, an inhibitor of thioredoxin in patients with advanced cancer of the pancreas following progression after gemcitabine-containing combination. *Cancer Chemother. Pharmacol.* 67 (3), 503–509.
- Reigan, P., Colucci, M.A., Siegel, D., Chilloux, A., Moody, C.J., Ross, D., 2007. Development of indolequinone mechanism-based inhibitors of NAD(P)H:quinone oxidoreductase 1 (NQO1): NQO1 inhibition and growth

- inhibitory activity in human pancreatic MIA PaCa-2 cancer cells. *Biochemistry* 46 (20), 5941–5950.
- Ren, D., Villeneuve, N.F., Jiang, T., Wu, T., Lau, A., Toppin, H.A., Zhang, D.D., 2011. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defence mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 108 (4), 1433–1438.
- Rivera, J.A., Rall, C.J., Graeme-Cook, F., Fernández-del Castillo, C., Shu, P., Lakey, N., Tepper, R., Rattner, D.W., Warshaw, A.L., Rustgi, A.K., 1997. Analysis of Kras oncogene mutations in CP with ductal hyperplasia. *Surgery* 121 (1), 42–49.
- Ross, D., Kepa, J.K., Winski, S.L., Beall, H.D., Anwar, A., Siegel, D., 2000. NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem. Biol. Interact.* 129 (1–2), 77–79.
- Rotblat, B., Melino, G., Knight, R.A., 2012. Nrf2 and p53: Januses in cancer? *Oncotarget* 3 (11), 1272–1283.
- Ryter, S.W., Alam, J., Choi, A.M., 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol. Rev.* 86 (2), 583–650.
- Sah, R.P., Garg, S.K., Dixit, A.K., Dudeja, V., Dawra, R.K., Saluja, A.K., 2014. Endoplasmic reticulum stress is chronically activated in chronic pancreatitis. *J. Biol. Chem.* 289 (40), 27551–27561.
- Satoh, H., Moriguchi, T., Taguchi, K., Takai, J., Maher, J.M., Suzuki, T., Winnard Jr., P.T., Raman, V., Ebina, M., Nukiwa, T., Yamamoto, M., 2010. Nrf2-deficiency creates a responsive microenvironment for metastasis to the lung. *Carcinogenesis* 31, 1833–1843.
- Selvendiran, K., Koga, H., Ueno, T., Yoshida, T., Maeyama, M., Torimura, T., Yano, H., Kojiro, M., Sata, M., 2006. Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. *Cancer Res.* 66 (9), 4826–4834.
- Shibata, T., Kokubu, A., Gotoh, M., Ojima, H., Ohta, T., Yamamoto, M., Hirohashi, S., 2008. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* 135 (4), 1358–1368.
- Siegel, D., Kepa, J.K., Ross, D., 2012. NAD(P)H:quinone oxidoreductase 1 (NQO1) localizes to the mitotic spindle in human cells. *PLoS ONE* 7 (9), e44861.
- Singh, A., Misra, V., Thimmulappa, R.K., Lee, H., Ames, S., Hoque, M.O., Herman, J.G., Baylín, S.B., Sidransky, D., Gabrielson, E., Brock, M.V., Biswal, S., 2006. Dysfunctional Keap1–Nrf2 interaction in non-small-cell lung cancer. *PLoS Med.* 3 (10), e420.
- Singh, S., Arcaroli, J., Thompson, D.C., Messermith, W., Vasiliou, V., 2015. Acetaldehyde and retinaldehyde-metabolizing enzymes in colon and pancreatic cancers. *Adv. Exp. Med. Biol.* 815, 281–294.
- Soini, Y., Eskelinen, M., Juvonen, P., Kärjälä, V., Haapasaari, K.M., Saarela, A., Karihtala, P., 2014. Nuclear Nrf2 expression is related to a poor survival in pancreatic adenocarcinoma. *Pathol. Res. Pract.* 210 (1), 35–39.
- Stevens, T., Berk, M.P., Lopez, R., Chung, Y.M., Zhang, R., Parsi, M.A., Bronner, M.P., Feldstein, A.E., 2012. Lipidomic profiling of serum and pancreatic fluid in chronic pancreatitis. *Pancreas* 41 (4), 518–522.
- Suzuki, T., Shibata, T., Takaya, K., Shiraiishi, K., Kohno, T., Kunitoh, H., Tsuta, K., Furuta, K., Goto, K., Hosoda, F., Sakamoto, H., Motohashi, H., Yamamoto, M., 2013. Regulatory nexus of synthesis and degradation deciphers cellular Nrf2 expression levels. *Mol. Cell. Biol.* 33 (12), 2402–2412.
- Taguchi, K., Fujikawa, N., Komatsu, M., Ishii, T., Unno, M., Akaike, T., Motohashi, H., Yamamoto, M., 2012. Keap1 degradation by autophagy for the maintenance of redox homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* 109 (34), 13561–13566.
- Tang, X., Wang, H., Fan, L., Wu, X., Xin, A., Ren, H., Wang, X.J., 2011. Luteolin inhibits Nrf2 leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs. *Free Radic. Biol. Med.* 50 (11), 1599–1609.
- Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. *J. Physiol.* 552, 335–344.
- Valenzuela, M., Glorieux, C., Stockis, J., Sid, B., Sandoval, J.M., Felipe, K.B., Kwiecinski, M.R., Verrax, J., Buc Calderon, P., 2014. Retinoic acid synergizes ATO-mediated cytotoxicity by precluding Nrf2 activity in AML cells. *Br. J. Cancer* 111 (5), 874–882.
- Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D.R., Harada, T., Engel, J.D., Yamamoto, M., 2003. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* 35 (3), 238–245.
- Wakabayashi, N., Dinkova-Kostova, A.T., Holtzclaw, W.D., Kang, M.I., Kobayashi, A., Yamamoto, M., Kensler, T.W., Talalay, P., 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response; fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. U. S. A.* 101 (7), 2040–2045.
- Wang, R., An, J., Ji, F., Jiao, H., Sun, H., Zhou, D., 2008. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem. Biophys. Res. Commun.* 373 (1), 151–154.
- Wang, X.J., Hayes, J.D., Henderson, C.J., Wolf, C.R., 2007. Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha. *Proc. Natl. Acad. Sci. U. S. A.* 104 (49), 19589–19594.
- Weinberg, F., Hamanaka, R., Wheaton, W.W., Weinberg, S., Joseph, J., Lopez, M., Kalyanaram, B., Mutlu, G.M., Budinger, G.R., Chandel, N.S., 2010. Mitochondrial metabolism and ROS generation are essential for K-ras mediated tumorigenicity. *Proc. Natl. Acad. Sci. U. S. A.* 107 (19), 8788–8793.
- Wenzel, U., Kuntz, S., Brendel, M.D., Daniel, H., 2000. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res.* 60 (14), 3823–3831.
- Whitcomb, D.C., Inflammation, Cancer, V., 2004. Chronic pancreatitis and pancreatic cancer. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287 (2), G315–G319.
- Yan, R., Zu, X., Ma, J., Liu, Z., Adeyanju, M., Cao, D., 2007. Aldo-keto reductase family 1 B10 gene silencing results in growth inhibition of colorectal cancer cells: implication for cancer intervention. *Int. J. Cancer* 121 (10), 2301–2306.
- Yan, C., Shieh, B., Reigan, P., Zhang, Z., Colucci, M.A., Chilloux, A., Newsome, J.J., Siegel, D., Chan, D., Moody, C.J., Ross, D., 2009. Potent activity of indolequinones against human pancreatic cancer: identification of thioredoxin reductase as a potential target. *Mol. Pharmacol.* 76, 163–172.
- Yan, C., Siegel, D., Newsome, J., Chilloux, A., Moody, C.J., Ross, D., 2012. Antitumor indolequinones induced apoptosis in human pancreatic cancer cells via inhibition of thioredoxin reductase and activation of redox signalling. *Mol. Pharmacol.* 81 (3), 401–410.
- Yang, L., Shen, J., He, S., Hu, G., Shen, J., Wang, F., Xu, L., Dai, W., Xiong, J., Ni, J., Guo, C., Wan, R., Wang, X., 2012. L-cysteine administration attenuates pancreatic fibrosis induced by TNBS in rats inhibiting the activation of pancreatic stellate cells. *PLoS ONE* 7 (2), e31807.
- Yi, Y.W., Oh, S., 2015. Comparative analysis of Nrf2-responsive gene expression in ACP-1 pancreatic cancer cell line. *Gene Genomics* 37, 97–109.
- Yu, S., Khor, T.O., Cheung, K.L., Li, W., Wu, T.Y., Huang, Y., Foster, B.A., Kan, Y.W., Kong, A.N., 2010. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. *PLoS ONE* 5 (1), e8579.
- Zeki, S., Miura, S., Suzuki, H., Watanabe, N., Adachi, M., Yokoyama, H., Horie, Y., Saito, H., Kato, S., Ishii, H., 2002. Xanthine oxidase-derived oxygen radicals play significant roles in the development of chronic pancreatitis in WBN/Kob rats. *J. Gastroenterol. Hepatol.* 17 (5), 606–616.
- Zhang, D.D., 2006. Mechanistic studies of the Nrf2–Keap1 signaling pathway. *Drug Metab. Rev.* 38 (4), 769–789.
- Zhang, D.D., Hannink, M., 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol.* 23 (22), 8137–8151.
- Zhang, D.D., Lo, S.C., Cross, J.V., Templeton, D.J., Hannink, M., 2004. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell. Biol.* 24 (24), 10941–10953.
- Zhang, K., Kaufman, R.J., 2008. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 454 (7203), 455–462.
- Zhang, P., Singh, A., Yegnasubramanian, S., Esopi, D., Kombairaju, P., Bodas, M., Wu, H., Bova, S.G., Biswal, S., 2010. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol. Cancer Ther.* 9 (2), 336–346.
- Zhang, W., Li, H., Yang, Y., Liao, J., Yang, G.Y., 2014. Knockdown or inhibition of aldo-keto reductase 1B10 inhibits pancreatic carcinoma growth via modulating Kras–E-cadherin pathway. *Cancer Lett.* 355 (2), 273–280.
- Zhang, Y., Yan, W., Collins, M.A., Bednar, F., Rakshit, S., Zetter, B.R., Stanger, B.Z., Chung, I., Rhim, A.D., di Magliano, M.P., 2013. Interleukin-6 is required for pancreatic cancer progression by promoting MAPK signalling activation and oxidative stress resistance. *Cancer Res.* 73 (20), 6359–6374.
- Zipper, L.M., Mulcahy, R.T., 2000. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochem. Biophys. Res. Commun.* 278 (2), 484–492.