Georg T. Wondrak *Editor*

Stress Response Pathways in Cancer

From Molecular Targets to Novel Therapeutics

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Editor
Georg T. Wondrak
Department of Pharmacology and Toxicology
College of Pharmacy & The University of Arizona Cancer Center
University of Arizona
Tucson, AZ, USA

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Preface

The biological revolution of the late twentieth century has fundamentally changed the way in which cancer is being understood, diagnosed, treated, and prevented; yet, it is now evident that the challenge to eliminate the suffering and death from cancer by 2015 issued in 2002 by Andrew von Eschenbach, then director of the National Cancer Institute, will not be met soon. I remember being in the audience, then a postdoctoral research fellow just having entered the cancer research arena, when attending my first AACR meeting held in San Francisco in 2002, where this challenge was made during a memorable plenary session with von Eschenbach serving as a keynote speaker. I left impressed and inspired, and even today, over 12 years later, it is the extraordinary boldness and exhilarating aspiration of the very possibility that this challenge might be fulfilled one day that overpowers the fact that this goal will not have been met by 2015. Ultimately, it is the grandness of our aspirations that determines how far we will go.

It is now understood that dysregulated cell stress response pathways play a critical role in tumorigenesis, and a refined mechanistic understanding of this phenomenon at the molecular level promises to open novel avenues for targeted therapeutic strategies that may benefit cancer patients in the near future. The comprehensive coverage of cell stress response pathways in cancer as presented for the first time in this book is intended to provide a state-of-the-art perspective that is of interest to both basic researchers focusing on fundamental cancer biology and translational biomedical health care professionals.

With the completion of this project I would like to express my gratitude to those who were instrumental in its creation. First, and foremost I would like to thank my co-authors from five continents that have graciously contributed their talent, expertise, and time to assemble this first-in-kind perspective on cancer stress response pathways. Secondly, I am indebted to my department head Terrence Monks for allowing me to pursue this project and to my friends and former postdoctoral mentors Mike and Elaine Jacobson for bringing me to San Francisco that day. Moreover, I am grateful for this outstanding opportunity and the expert support provided by Melania Ruiz and Ilse Hensen-Kooijman at Springer Science + Business Media B.V.

Finally, I would like to thank my family, Claudia, Gil, Philip, and Annie, for letting me divert precious time and energy from them in pursuit of this book project and for sharing my hope that the research presented here will move us closer to take on von Eschenbach's challenge one day.

Tucson, AZ, USA Georg T. Wondrak
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Contents

Contributors

Fabienne Aillet Inbiomed, Parque Tecnológico de Miramón, San Sebastian, Gipuzkoa, Spain

Andrea Alimonti Institute of Oncology Research (IOR), Oncology Institute of Southern Switzerland (IOSI), Bellinzona, Switzerland

Tasleem Arif Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Subhasree Basu Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, USA

Lili Belcastro Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, USA

Danya Ben-Hail Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Laia Bosch-Presegué Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain

Anna Budina-Kolomets Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, USA

Amancio Carnero Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio, Consejo Superior de Investigaciones Cientificas (CSIC), Universidad de Sevilla, Sevilla, Spain

C. Ross Carter Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK

Robert Clarke Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Diletta Di Mitri Institute of Oncology Research (IOR), Oncology Institute of Southern Switzerland (IOSI), Bellinzona, Switzerland

Brian Gabrielli The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Woolloongabba, Brisbane, QLD, Australia

Shanmugasundaram Ganapathy-Kanniappan Division of Interventional Radiology, Department of Radiology and Radiological Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Luciana E. Giono Laboratorio de Fisiología y Biología Molecular, Departamento de Fisiología y Biología Molecular, IFIBYNE, UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Ciudad de Buenos Aires, Argentina

Shaan Gupta Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Nikolas K. Haass The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Woolloongabba, Brisbane, QLD, Australia

Nigel B. Jamieson Academic Department of Surgery, College of Medical, Veterinary, and Life Sciences, University of Glasgow, Glasgow, UK

Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK

Madhuri Kalathur Institute of Oncology Research (IOR), Oncology Institute of Southern Switzerland (IOSI), Bellinzona, Switzerland

M. Fátima Ladelfa Laboratorio de Biología Celular y Molecular, Departamento de Química Biológica, IQUIBICEN, UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Ciudad de Buenos Aires, Argentina

Valérie Lang Inbiomed, Parque Tecnológico de Miramón, San Sebastian, Gipuzkoa, Spain

Jill E. Larsen QIMR Berghofer Medical Research Institute, Herston, Brisbane, QLD, Australia

Haoran Li Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Zihai Li Department of Microbiology and Immunology, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA

Bei Liu Department of Microbiology and Immunology, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA

Sofía Lobato-Gil Inbiomed. Paseo Mikeletegi 81, Parque Tecnológico de Miramón, Gipuzkoa, Spain

Navin R. Mahadevan Department of Medicine and Moores Cancer Center, The Laboratory of Immunology, University of California, San Diego, La Jolla, CA, USA

Lydia Mata-Cantero Inbiomed, Paseo Mikeletegi 81, Parque Tecnológico de Miramón, Gipuzkoa, Spain

Centro de Investigación Básica (CIB), GlaxoSmithKline, Tres Cantos, Madrid, Spain

Dario Mizrachi School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, USA

Martín Monte Laboratorio de Biología Celular y Molecular, Departamento de Química Biológica, IQUIBICEN, UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Ciudad de Buenos Aires, Argentina

Sandra Muñoz-Galvan Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio, Consejo Superior de Investigaciones Cientificas (CSIC), Universidad de Sevilla, Sevilla, Spain

Maureen E. Murphy Program in Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, USA

Jac A. Nickoloff Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO, USA

Zay Yar Oo The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Woolloongabba, Brisbane, QLD, Australia

Sandra Pavey The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Woolloongabba, Brisbane, QLD, Australia

Kristopher S. Raghavan Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Manuel S. Rodriguez Inbiomed, Parque Tecnológico de Miramón, San Sebastian, Gipuzkoa, Spain

Jeffrey J. Rodvold Department of Medicine and Moores Cancer Center, The Laboratory of Immunology, University of California, San Diego, La Jolla, CA, USA

Ayesha N. Shajahan-Haq Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

Varda Shoshan-Barmatz Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Anna Shteinfer Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Núria Sima Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain

Loredana Spoerri The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Woolloongabba, Brisbane, QLD, Australia

Colin W. Steele Beatson Institute for Cancer Research, Glasgow, UK

Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK

Ceen-Ming Tang Department of Pharmacology, University of Oxford, Oxford, UK

Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, Institute of Digestive Disease, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, Hong Kong

Alejandro Vaquero Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain

Georg T. Wondrak Department of Pharmacology and Toxicology, College of Pharmacy and The University of Arizona Cancer Center, University of Arizona, Tucson, AZ, USA

Burton B. Yang Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, Toronto, Canada

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

Jun Yu Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, Institute of Digestive Disease, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, Hong Kong

Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong

Maurizio Zanetti Department of Medicine and Moores Cancer Center, The Laboratory of Immunology, University of California, San Diego, La Jolla, CA, USA

Chapter 1
Introduction to Cell Stress Responses in Cancer: The Big Picture

Georg T. Wondrak

Abstract Cancer represents a major medical challenge of our time, exacerbated by the paradoxical combination of an age-related increase in cancer incidence and a demographic shift towards older populations worldwide. During the last 10 years, significantly improved strategies for molecular cancer prevention and targeted therapeutic intervention have become available, an encouraging sign indicating that decades of global commitment to biomedical research, implementing what is now known as the biological revolution of the late twentieth century, start bearing fruit for cancer patients and survivors. It has now become apparent that oncogene-driven tumorigenesis is associated with specific stress phenotypes including DNA damage stress, mitotic stress, metabolic stress, proteotoxic stress, and oxidative stress. Importantly, cancer cells depend on the counter-regulatory activation of cytoprotective stress response pathways enabling adaptive capabilities that antagonize the cytotoxic consequences of oncogenesis-associated cellular stresses, and cumulative research indicates that the essential nature of these stress response pathways represents a specific molecular vulnerability amenable to therapeutic intervention targeting this emerging Achilles heel of malignancy.

Keywords Hallmarks of cancer • DNA damage stress • Mitotic stress • Metabolic stress • Proteotoxic stress • Oncogene-driven tumorigenesis • Oxidative stress • Cancer • Molecular vulnerability

1.1 Cancer 'Stress Phenotypes': From Molecular Mechanisms to Therapeutic Opportunities

Cancer represents a major medical challenge of our time, exacerbated by the paradoxical combination of an age-related increase in cancer incidence and a demographic shift towards older populations worldwide. During the last 10 years,

G.T. Wondrak (✉)
Department of Pharmacology and Toxicology, College of Pharmacy and The University of Arizona Cancer Center, University of Arizona, 1515 N. Campbell Ave., Tucson, AZ 85724, USA
e-mail: wondrak@pharmacy.arizona.edu

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significantly improved strategies for molecular cancer prevention and targeted therapeutic intervention have become available, an encouraging sign indicating that decades of global commitment to biomedical research, implementing what is now known as the biological revolution of the late twentieth century, start bearing fruit for cancer patients and survivors. Even though molecular understanding, diagnosis, treatment, and prevention of this disease have been revolutionized, cancer still represents an ultimate frontier to be conquered only by sustained future research efforts and investments.

In a seminal review published in 2000, Hanahan and Weinberg proposed that 'the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth' (Hanahan and Weinberg 2000). The six hallmarks of cancer encompass self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. This original set was expanded subsequently by inclusion of additional hallmarks of potential generality, including the evasion of immune destruction and metabolic reprogramming (Kroemer and Pouyssegur 2008; Hanahan and Weinberg 2011). Furthermore, the concept of a specific tumor microenvironment provided by functionally altered (yet not transformed) bystander cells that are instrumental to the acquisition of other hallmark traits, such as invasiveness, was proposed as another independent signature hallmark. Remarkable conceptual progress towards understanding the mechanistic framework underlying each molecular hallmark has been achieved over the last decade, and it has now become apparent that oncogene-driven tumorigenesis is associated with five additional hallmarks referred to jointly as cancer 'stress phenotypes': DNA damage stress, mitotic stress, metabolic stress, proteotoxic stress, and oxidative stress (Luo et al. 2009).

Importantly, cancer cells depend on the counter-regulatory activation of cytoprotective stress response pathways enabling adaptive capabilities that antagonize the cytotoxic consequences of oncogenesis-associated cellular stresses, and cumulative research indicates that the essential nature of these stress response pathways represents a specific molecular vulnerability amenable to therapeutic intervention targeting this emerging Achilles heel of malignancy.

For example, pharmacological modulation of proteotoxic stress has recently emerged as a promising strategy for chemotherapeutic intervention targeting cancer cells (Obeng et al. 2006; Healy et al. 2009; De Raedt et al. 2011; Qiao et al. 2012). Proteotoxic stress occurs in response to cytotoxic stimuli that cause accumulation of unfolded and/or misfolded proteins including heat shock, oxidative stress, calcium dysregulation, and proteasome inhibition. It is now widely accepted that tumor cells are exposed to high levels of endogenous proteotoxic stress originating from mutation-driven expression of misfolded proteins and adverse conditions associated with the tumor microenvironment including hypoxia, energy crisis, and redox dysregulation (Dai et al. 2012). In addition, certain oncogene-encoded proteins (e.g. $Braf^{V600E}$ in malignant melanoma cells) depend on heat shock protein (Hsp90)-mediated stabilization that prevents their rapid turnover and inactivation (Grbovic et al. 2006). Therefore, pharmacological modulation of proteotoxic stress (by targeting

the cellular heat shock or unfolded protein responses, the ubiquitin-proteasome system, or the autophagic-lysosomal proteolytic machinery) may trigger preferential cytotoxicity in cancer cells without compromising viability of normal cells displaying lower constitutive levels of endogenous proteotoxic stress.

The phenotypic stress hallmarks of cancer and their enabling stress response pathways, as proposed in a seminal review article published in 2009, are characterized by extensive functional crosstalk and complex networks of mechanistic interdependence (Luo et al. 2009). For example, cancer cell aneuploidy originating from oncogene-driven replication stress and mitotic aberrations causes expression of dysfunctional proteins underlying proteotoxic stress that must be counterbalanced by the upregulation of specific cytoprotective pathways such as the heat shock stress response and the ubiquitin-proteasome system. Likewise, elevated levels of reactive oxygen species originating from dysregulated redox signaling and mitochondrial electron leakage will result in increased levels of oxidative DNA damage triggering cellular senescence or apoptosis unless counterbalanced by altered genotoxic, redox, and other stress response pathways enabling cancer cell survival and proliferation.

1.2 Pushing Cancer Cells 'Off the Cliff'

A painting depicting The Cliff at Dieppe, Normandy as created by Claude Monet in 1882, illustrates the general concept that oncogene-driven and other alterations enhancing tumorigenic performance may at the same time cause specific molecular vulnerabilities and dependencies that can be harnessed for therapeutic intervention (Fig. 1.1). The house standing closer to the cliff (photoshopped out of its original position away from the main building as indicated by the red square) has acquired the ability to stand under intrinsically unstable conditions and will now become more dependent on the firmness and solidity of the ground. However, since residing closer to the edge, minor adverse occurrences (such as a small landslide after an extended period of rain undermining the firmness of the sandy edges; red arrows) will be sufficient to push the house off the cliff, whereas the main building stands firm under stressful conditions. In the same way, targeted molecular interventions that modulate certain components and functions of the cancer cell stress machinery will preferentially push cancer cells 'off the cliff' thereby undermining tumorigenesis (Wondrak 2009; Chan and Giaccia 2011; Raj et al. 2011; Shaheen et al. 2011; Benbrook and Long 2012).

In this book entitled 'Stress Response Pathways in Cancer', leading authorities in research institutions from five continents have contributed 17 chapters that cover the complex framework underlying alterations of cell stress response pathways in tumorigenesis focusing on molecular mechanisms and potential therapeutic opportunities. The chapters center on the following areas of cancer stress regulation: Coverage of DNA damage stress response (*Chap. 2*) and cell cycle checkpoint dysregulation (*Chap. 3*) is followed by a comprehensive review of p53 function in

Fig. 1.1 Pushing cancer cells 'off the cliff'. Cliff at Dieppe by Claude Monet, 1882 (Adapted from http://www.wikiart.org/en/claude-monet/cliff-at-dieppe; public domain)

stress response and tumorigenesis (*Chap. 4*). Next, sirtuins are presented as a double-edged sword in cancer (*Chap. 5*), followed by an introduction to microRNA-regulated stress responses as a novel causative factor in tumorigenesis (*Chap. 6*). The following two chapters are dedicated to the complex role of cellular senescence, covering its mechanistic role in oncogenesis (*Chap. 7*) and presenting therapeutic opportunities of pro-senescent intervention (*Chap. 8*). The important role of proteotoxic stress in tumorigenesis and cancer therapy is explored extensively in the following chapters. First, the integrated proteotoxic stress response is interrogated for induction of cancer cell apoptosis (*Chap. 9*), followed by an in-depth coverage of Hsp70 heat shock protein family function in tumorigenesis (*Chap. 10*). Next, a comprehensive exploration of the ubiquitin-proteasome system (UPS) as an important cancer drug target is undertaken (*Chap. 11*). The role of endoplasmatic reticulum (ER) stress in tumorigenesis is presented focusing on molecular mechanisms and therapeutic opportunities in multiple myeloma (*Chap. 12*). Next, the role of oxidative stress and redox dysregulation in tumorigenesis is covered focusing on genetic alterations in malignant melanoma (*Chap. 13*). The following three chapters of the book illuminate the important subject of tumorigenic metabolic reprogramming. First, molecular mechanisms and therapeutic opportunities associated with hypoxic adaptations of cancer cells are explored (*Chap. 14*). Next, the tumorigenic function of glycolytic adaptations and their potential role for therapeutic intervention are covered comprehensively (*Chap. 15*). Finally, the role of VDAC1 as a promising cancer drug target based on its function in mitochondrial

metabolite transport and apoptosis is presented (*Chap. 16*). Finally, a chapter is dedicated to the important role of inflammatory dysregulation in tumorigenesis with a focus on pancreatic cancer (*Chap. 17*), followed by an integrative coverage of the novel concept of cell-nonautonomous ER stress-mediated dysregulation of immune function by cancer cells (*Chap. 18*).

The comprehensive coverage of cell stress response pathways in cancer presented in this multi-author book is intended to provide a state-of-the-art perspective that is of interest to both basic researchers focusing on fundamental cancer biology and translational biomedical health care professionals. Most importantly, it is to be hoped that the ultimate outcome by which to measure success of this project will be its ability to build promising avenues to improved therapeutic interventions that benefit both cancer patients and survivors in the near future.

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Chapter 2
DNA Repair Dysregulation in Cancer: From Molecular Mechanisms to Synthetic Lethal Opportunities

Jac A. Nickoloff

Abstract Targeted cancer therapies have excellent potential for increasing long-term patient survival while minimizing short- and long-term side effects of therapy including neurological problems and secondary cancers. DNA repair systems suppress genome instability that drives the acquisition of tumorigenic mutations. Most cancer therapeutics cause DNA damage, yet despite having DNA repair defects, tumors often display resistance to therapy because redundant repair pathways can process DNA damage efficiently. By targeting the redundant repair pathways, tumors can be sensitized to endogenous and/or exogenous DNA damage, described as synthetic lethality or synthetic sensitivity. There have been notable successes in applying synthetic lethal and sensitive approaches to treat cancer, but challenges remain as tumors can acquire resistance due to their rapid evolution driven by ongoing genome instability. It is important to improve our understanding of DNA repair pathways to better exploit tumor weaknesses imparted by DNA repair defects.

Keywords DNA damage • DNA double-strand break repair • Non-homologous end-joining • Homologous recombination • Nucleotide excision repair • Base excision repair • Mismatch repair • Mutagenesis • DNA replication stress • Targeted cancer therapy • Synthetic lethality • Genome instability

2.1 Introduction

DNA repair pathways play critical roles in cancer suppression, etiology, and therapy. DNA damage is ubiquitous, and organisms have evolved sophisticated mechanisms to repair the many types of DNA lesions that arise spontaneously and that are induced by exogenous genotoxins including radiation and reactive chemicals. DNA repair pathways play important roles in the accurate transmission of the

J.A. Nickoloff (✉)
Department of Environmental and Radiological Health Sciences,
Colorado State University, Fort Collins, CO 80523, USA
e-mail: J.Nickoloff@colostate.edu

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genome to daughter cells, a central feature of cell division. However, DNA repair is principally designed to restore the *chemical* integrity of DNA without regard for restoring *genetic* integrity, hence repair of DNA damage is sometimes associated with genetic change, i.e., mutation, ranging from single-base substitutions to chromosomal rearrangements. These changes may be detrimental, leading to cancer or other genetic diseases, but they also play important roles in immune system development and evolution.

There are five main classes of DNA repair pathways, each with multiple subpathways. For the most part, specific types of DNA lesions are repaired by a specific pathway/subpathway, but if repair fails, the lesion may be shunted to a secondary (redundant) pathway. In addition, it is often the case that particular steps along a DNA repair pathway create other forms of damage (i.e., single-strand breaks) that require repair. Thus, the constellation of DNA repair pathways function in complex networks. The redundancy inherent in these networks creates robust systems that maintain genome stability and confer resistance to the cytotoxic effects of DNA damaging agents. This is beneficial in normal cells and tissues, but resistance of cancer cells to radio- and chemotherapy presents significant challenges to oncologists. A deep understanding of DNA repair mechanisms and pathway redundancy can reveal weaknesses in cancer cells that can be exploited to improve therapeutic outcomes. Thus, cancer cells with a defect in a primary DNA repair pathway may be dependent on a redundant, secondary pathway for survival. In this case, inhibiting the secondary pathway is lethal to cancer cells, but not normal cells which retain the functional primary pathway (Fig. 2.1a, b). If the damage processed by these pathways arises spontaneously, the lethal combination ("synthesis") of genetic mutations (and/or inhibited targets) in redundant pathways is termed "synthetic genetic lethality" or simply "synthetic lethality." If damage is induced by a genotoxin,

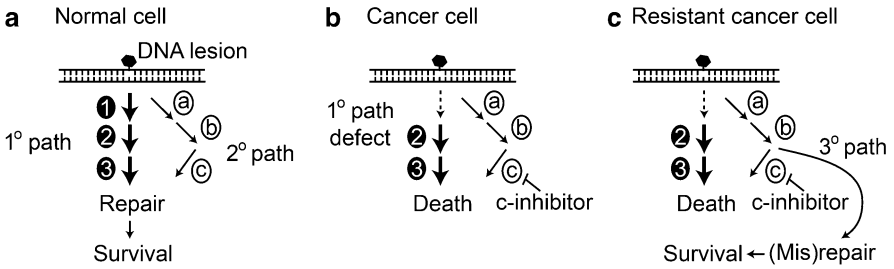

Fig. 2.1 Synthetic lethality. (**a**) Idealized DNA repair pathways in a normal cell that process a specific DNA lesion, with primary and secondary pathways catalyzed by enzymes 1–3 or a–c, respectively. (**b**) Cancer cell lacking enzyme 1 shifts repair from the primary to secondary pathway. Inhibition (or mutation) of any step in the secondary pathway is synthetically lethal. (**c**) Activation of tertiary pathway rescues the cell from synthetic lethality, producing a cancer cell resistant to the inhibitor. The tertiary pathway may be error-prone; the cell may survive the damage but with increased mutation load (genome instability) due to misrepair, which can drive tumor progression and further resistance to subsequent therapy

rather than arising spontaneously, these types of genetic interactions are properly described as conferring "synthetic sensitivity" to the genotoxin. While therapeutic approaches based on synthetic lethality and sensitivity clearly have merit, it is important to note that tertiary repair pathways may exist, or they may arise or be activated through other mutations, allowing cancer cells to evade synthetic lethality or sensitivity (gain resistance) (Fig. 2.1c). The promise of synthetic lethality and synthetic sensitivity hinges on detailed knowledge of DNA repair pathways for the design of effective targeted cancer therapies.

2.2 DNA Repair Mechanisms

2.2.1 Base Excision Repair, Nucleotide Excision Repair, and Mismatch Repair

There are five classes of DNA repair pathways, each with two or more subtypes (Fig. 2.2). Three sets of pathways act on damage present on single strands. Base excision repair (BER) comprises several subpathways that repair non-bulky DNA lesions such as ring-opened bases and small adducts like oxidized bases (Fig. 2.2a). The first step in BER is lesion recognition by one of several glycosylases that remove the damaged base, producing an abasic site. PARP1, APE1 endonuclease, and deoxyribophosphodiesterase activities create a single-strand break and DNA repair is completed by DNA polymerase and DNA ligase activities (Krokan and Bjoras 2013). Nucleotide excision repair (NER) processes bulky lesions that distort the double helix, including pyrimidine dimers and large DNA adducts Kamileri et al. 2012) (Fig. 2.2b). NER requires lesion recognition proteins and endonucleases that create single-strand breaks ~15 nt from the lesion, a helicase removes the ~30 nt oligonucleotide carrying the lesion, DNA polymerase then fills the single-strand gap and ligase completes the repair. Mismatch repair (MMR) differs from all other DNA repair pathways in that there is no "damage" per se, but instead chemically intact mismatched bases occur on otherwise complementary strands (Hsieh and Yamane 2008). Mismatches arise via replication errors, strand exchange during homologous recombination (HR), and deamination of 5′-methyl cytosine which produces thyimidine and a G-T mismatch. MMR also processes single- and multi-base loops that arise when bases are inserted or deleted – insertion/deletion loop mismatches can arise by replication errors (especially in sequences with mononucleotide repeats, or certain trinucleotide repeats that are prone to self-annealing as these form relatively stable hairpin structures), or during homologous recombination (see below). MMR involves mismatch recognition, single-strand nicking 5′ and/or 3′ of the mismatch from which long-tract single-strand excision removes the mismatched base(s), re-synthesis to fill the single-strand gap, and ligation (Fig. 2.2c). Long-tract MMR operates on mismatches that arise during replication and HR; G-T mismatches can be repaired by long-tract MMR or by a specialized G-T MMR

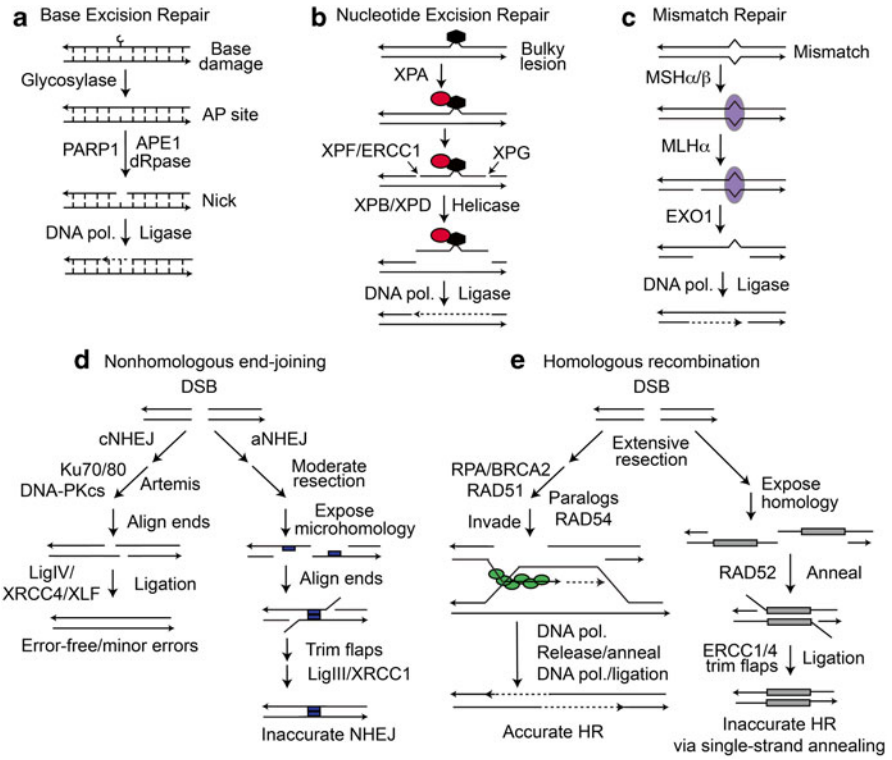

Fig. 2.2 DNA repair pathways. (**a**) Repair of base damage by BER results in short patch repair and is promoted by PARP1. (**b**) Bulky lesion repair by NER removes a ~30 nt single strand oligonucleotide carrying the lesion. (**c**) MMR involves long-patch excision and resynthesis initiated at nicks distant from the mismatch. (**d**) NHEJ includes relatively accurate cNHEJ, and inaccurate aNHEJ pathways distinguished by the extent of end resection, requirement for microhomology (*blue boxes*). (**e**) HR catalyzed by RAD51 (*green ovals*) is generally accurate. BRCA1 and FANC proteins (not shown) also function in RAD51-dependent HR. SSA between linked repeats (*grey boxes*) deletes one repeat and intervening sequences

system that is more akin to BER as it is initiated by a G-T specific glycosylase (Bill et al. 1998; Wiebauer and Jiricny 1990). BER, NER, and MMR are relatively accurate repair mechanisms because each uses an intact complementary strand opposite the lesion to direct repair. However, repair polymerases tend to be less accurate than replicative polymerases, and this can result in localized mutagenesis. In addition, BER proceeds through abasic intermediates that can be subject to translesion DNA synthesis by Y-family polymerases, which are low processivity, error-prone DNA polymerases, providing another path to localized mutagenesis (Simonelli et al. 2005). There are also examples where the accuracy of DNA repair systems are downregulated to specifically enhance mutagenesis, e.g., MMR induced trinucleotide repeat expansion and antibody maturation (Pena-Diaz and Jiricny 2012).

2.2.2 Features and Roles of DSBs and DSB Repair Pathways

DSBs are the most important DNA lesion because they can trigger genome rearrangements and unrepaired DSBs are usually lethal. DSBs are induced by ionizing radiation, and by endogenous nucleases during meiosis (SPO11) and V(D)J recombination (RAG1/2) (Keeney and Neale 2006; Nishana and Raghavan 2012). AID deaminates cytosine to uracil which can be processed to staggered single-strand breaks to create DSBs that trigger immunoglobulin class switch recombination or gene conversion (Daniel and Nussenzweig 2013), which along with V(D)J recombination are important mechanisms for generating antibody diversity. DSBs also arise during DNA replication when forks encounter blocking lesions (single-strand breaks, many types of base damage, most DNA adducts, pyrimidine dimers, and intra- and inter-strand crosslinks) (Allen et al. 2011; Budzowska and Kanaar 2009). DSBs are marked by phosphorylated histone H2AX (γ-H2AX) (Ward and Chen 2001) which plays important roles in DNA damage checkpoint signaling and DSB repair (Chanoux et al. 2008; Downey and Durocher 2006).

DSBs are repaired by nonhomologous end-joining (NHEJ) and HR (Fig. 2.2d, e). DSB repair by NHEJ is frequently inaccurate, yielding short (1–20 nt) deletion or insertion mutations (Deriano and Roth 2013). When two DSBs occur simultaneously on different chromosomes, NHEJ can mediate translocations (Lieber et al. 2006; Nickoloff et al. 2008; Weinstock et al. 2006). HR is generally accurate, but since HR can occur between any two homologous sequences (sister chromatids, homologous chromosomes, linked repeats in inverted or direct orientation, and repeats on non-homologous chromosomes), HR poses significant risks of medium- to large-scale genome rearrangements including deletions, inversions, amplifications, small- to large-scale loss of heterozygosity (LOH), and translocations (Nickoloff 2002).

2.2.3 DSB Repair by Nonhomologous End-Joining

NHEJ comprises two pathways, classical and alternative NHEJ (cNHEJ, aNHEJ) (Fig. 2.2d). Although both pathways are inaccurate, cNHEJ is more accurate and is the dominant DSB repair pathway in mammalian cells; aNHEJ appears to serve as a backup pathway to cNHEJ as it is typically observed when cells have a defect in a cNHEJ factor (Deriano and Roth 2013; Weinstock et al. 2007; Wray et al. 2010, 2013). cNHEJ involves little to no end-resection, whereas moderate end-resection is key to exposing microhomologies on complementary strands central to the aNHEJ pathway (aNHEJ is sometimes called "microhomology-mediated end-joining"). 53BP1 and BRCA1 are implicated in regulation of end-resection, which in turn regulates cNHEJ – aNHEJ pathway choice (and NHEJ – HR choice; see below) (Deriano and Roth 2013; Panier and Boulton 2014; Symington and Gautier 2011).

cNHEJ initiates with Ku70/Ku80 binding to DSB ends to which DNA-PKcs is recruited, activating its kinase. Artemis is a nuclease required for processing certain

types of broken ends (Jacobs et al. 2010). Metnase is a recently evolved nuclease and protein methylase that promotes cNHEJ by enhancing recruitment and retention of other NHEJ factors by methylating histone H3 (Fnu et al. 2011), and possibly through nucleolytic end processing (Hromas et al. 2008). Together these factors promote association of broken ends with little to no base-pairing (Lieber 2010). Prior to ligation by Ligase IV and accessory factors XRCC4 and XLF, DNA-PKcs phosphorylation by itself or ATM stimulates its dissociation from ends (Dahm 2008; Lieber 2010). Genetic defects in, or inhibition of, cNHEJ factors shunts DSBs to aNHEJ, which depends on moderate end-resection by MRE11-RAD50-NBS1 (MRN) and CtIP to expose microhomologies, and requires PARP1 and Ligase III-XRCC1 (Deriano and Roth 2013; Rupnik et al. 2010; Wray et al. 2013; You et al. 2009). By suppressing resection by MRN and CtIP, 53BP1 prevents aNHEJ and thus reduces the risk of aNHEJ-mediated translocations (Bothmer et al. 2010). PARP1 inhibitors also prevent aNHEJ-mediated translocations, and may be useful in reducing the risk of oncogenic translocations associated with cancer chemotherapy (Wray et al. 2013).

2.2.4 DSB Repair by Homologous Recombination

HR comprises two pathways, a conservative, accurate pathway that involves strand invasion mediated by RAD51, and a non-conservative, error-prone, RAD51-independent pathway termed single-strand annealing (SSA) (Fig. 2.2e). Both HR pathways require extensive end-resection (100–1,000s of bases). Cells with defects in RAD51-dependent HR shunt DSBs to SSA, destabilizing the genome (Tutt et al. 2001). DSB repair pathway choice is apparently regulated by proteins that control resection, including 53BP1 and BRCA1 (Panier and Boulton 2014), with increasing resection along a cNHEJ – aNHEJ – HR continuum. The extensive resection required for HR begins with end-processing by MRN-CtIP followed by BLM helicase and two nucleases, DNA2 and EXO1 (Symington and Gautier 2011). RAD51-dependent HR can lead to localized LOH termed gene conversion, a mechanism that nearly always results in unidirectional (non-reciprocal) transfer of information from an unbroken donor molecule to a broken homologous molecule during DSB repair – the LOH region is termed a gene conversion tract and these can range from just a few bp to many kbp. Gene conversions are sometimes associated with reciprocal exchange of sequences flanking the conversion tract, and during HR between homologous chromosomes, 50 % of crossovers can cause LOH of an entire chromosome arm, extending from the point of the crossover to the telomere (Nickoloff 2002). In cells with defects in HR proteins, RAD51-mediated strand exchange may abort after the strand invasion and repair synthesis is initiated but before second end capture. In this case, synthesis continues to the end of the donor chromosome, which also results in LOH from the DSB to the telomere in a process termed “break-induced replication” (Llorente et al. 2008). As noted above, crossovers also pose significant risk

of large-scale genome rearrangement, and are suppressed by proteins like BLM (Cheok et al. 2005). RAD51-dependent HR is generally restricted to S and G2 phases when sister chromatids serve as closely associated, essentially 100 % accurate repair templates with low risk of large-scale genome rearrangement.

End resection produces long, 3′ ssDNA tails bound by RPA that is exchanged for RAD51 in a reaction promoted by "mediator" proteins BRCA2, RAD52, and RAD51 paralogs (XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D). The RAD51-ssDNA nucleoprotein filament searches for and invades homologous sequences, the invading strand is extended by DNA polymerase, and then released and "captured" by the resected end on the opposite side of the DSB. This one-ended invasion mechanism, termed synthesis-dependent strand annealing (SDSA), poses low risk of crossovers (Fig. 2.2e). In some cases, both ends invade, creating a double Holiday junction intermediate that is resolved by BLM-TOP3α-RMI1-2 without crossing over (Heyer et al. 2010). In the absence of BLM, crossovers are much more frequent and this confers a genome-instability and cancer-prone phenotype (Cheok et al. 2005). Some RAD51 mediator proteins also function later in HR, stabilizing the strand invasion intermediate and removing RAD51 from ssDNA after the RAD51-ssDNA filament invades the homologous sequence to prepare for repair synthesis (Brenneman et al. 2002; Fortin and Symington 2002). RAD54 (and possibly RAD54B) also act late, altering chromatin in donor sequences to promote strand invasion (Heyer et al. 2010).

The SSA pathway (Fig. 2.2e) is RAD51-independent and at least in yeast, depends on the strand annealing activity of RAD52 (Heyer et al. 2010). SSA involves extensive resection to expose complementary sequences in linked, direct repeats which anneal to produce a deletion product lacking one of the repeats and the intervening sequences. SSA can also mediate translocations (Weinstock et al. 2006). SSA and RAD51-mediated gene conversion are competing HR pathways, and the balance shifts toward SSA for closely spaced repeats (Schildkraut et al. 2005).

2.2.5 Role of HR in Replication Stress Responses

Nearly all DNA lesions, whether induced by chemotherapeutics or ionizing radiation, block replicative DNA polymerases, causing "replication stress." RAD51-mediated HR plays a critical role in restarting stalled and collapsed replication forks, and it is this role that accounts for the fact that RAD51 is an essential protein in mammalian cells (Allen et al. 2011). When replication forks encounter blocking lesions, the fork stalls and the replisome is stabilized by DNA repair and checkpoint proteins including RPA, ATR-ATRIP, ATM, BLM, and INO80 (Budzowska and Kanaar 2009; Davies et al. 2007; Shimada et al. 2008; Zou et al. 2006). If a stalled fork is not restarted in timely manner, it collapses to a DSB. Unlike DSBs induced by nucleases or ionizing radiation, fork collapse yields single-ended DSBs that cannot be readily repaired by NHEJ. Like two-ended DSBs, one-ended DSBs are

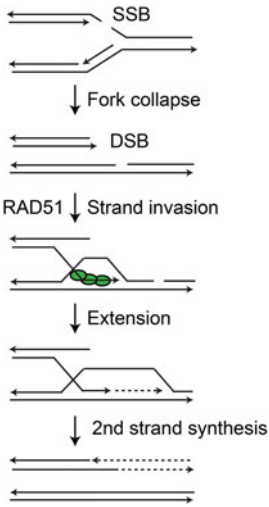

Fig. 2.3 Restart of collapsed replication fork. Fork encounter with lesion (single-strand break in this example) causes collapse to one-ended DSB. RAD51 loads onto resected 3′ end that invades the sister chromatid, is extended, and then serves as template for second strand synthesis to complete DNA replication

marked by γ-H2AX, and the induction and resolution of this signal are measures of fork collapse and repair (De Haro et al. 2010; Kim et al. 2014). Stalled and collapsed replication forks can be restarted by several HR-related mechanisms (Allen et al. 2011; Budzowska and Kanaar 2009); one mechanism is shown in Fig. 2.3. A fork stalled by a blocking lesion can "regress", and this allows the lesion to be bypassed via an HR mechanism (Budzowska and Kanaar 2009). Lesion bypass via fork regression is not a repair mechanism, but a damage tolerance mechanism. Another type of damage tolerance mechanism is mediated by error-prone, translesion synthesis (TLS) polymerases (Sale 2013). Understanding tumor cell responses to replication stress, and in particular, the role of checkpoint and HR pathways in these responses, has emerged as a critical topic in cancer biology.

2.3 DNA Repair Pathway Regulation and Networks

DNA repair pathways are highly regulated and exist in complex, interacting networks. DNA repair can be regulated in many ways. For example, repair proteins may be absent (gene knockout) or exist as hypomorphs with reduced or altered function; mRNA levels can be altered by transcription factors, microRNAs, and other factors that regulate mRNA stability; and protein stability and function can be altered by posttranslational modifications such as phosphorylation, ubiquitylation, and SUMOylation. There is evidence that oxidative damage upregulates both BER and NER (Cabelof et al. 2002; Kirkali et al. 2011), and gene expression profiling has shown that DNA damage upregulates many DNA repair genes (Friedberg et al. 2005). At a functional level, exposure of cells to low levels of DNA damage confers resistance to a subsequent higher dose, so-called adaptive responses (Huang et al. 2006;

Preston 2005). Adaptive responses are protective, which is beneficial for normal tissue, but can have the negative effect of conferring resistance of the tumor to treatment. Adaptive responses may reflect broad effects of damage on repair, checkpoint, and programmed cell death pathways, rather than upregulation of specific DNA repair pathways. For example, there is no clear evidence for upregulation of HR by DSB damage (Heyer et al. 2010), yet adaptive responses to ionizing radiation have been described (Preston 2005).

As noted above, DSB repair pathway choice is regulated by resection of DSB ends in a cell cycle dependent manner (Durant and Nickoloff 2005; Shrivastav et al. 2008; Symington and Gautier 2011). The upregulation of HR during S and G2 phase probably reflects the combined effects of end resection and availability of sister chromatid repair templates. When considering synthetic lethal or sensitivity approaches to cancer therapy, DSB repair illustrates an important principal: a single type of lesion can be shunted to multiple repair pathways that operate in hierarchical fashion (Fig. 2.1). In the case of DSBs, this hierarchy runs from cNHEJ to aNHEJ to HR. Moreover, DNA repair pathways display considerable functional overlap. As just one example of network connectivity, NER has known functional interactions with MMR, BER, HR, NHEJ, and TLS (Shaheen et al. 2011). There are several types of interactions among repair pathways, including shared repair factors, processing of specific lesions by multiple pathways, and common repair intermediates produced by different pathways.

DNA repair pathways are also tightly integrated with DNA damage checkpoint pathways. DNA damage checkpoints were originally defined as damage sensor, signaling and effector pathways that arrested cells in specific phases of the cell cycle, ostensibly to allow time for repair before resuming the cell cycle to reduce problems associated with replication or segregation of damaged DNA. However, studies in yeast demonstrated that artificially arresting certain checkpoint-defective mutants failed to rescue damage sensitivity (DeMase et al. 2005; Redon et al. 2003; Toh et al. 2006). There is now clear evidence that checkpoint factors, such as ATM and γ-H2AX regulate both checkpoints and DNA repair (Downey and Durocher 2006; Shrivastav et al. 2009; Smith et al. 2010; Xie et al. 2004).

The tumor microenvironment imposes considerable stress on tumor cells, including glucose and oxygen deprivation, low pH, replication stress associated with activated oncogenes, and of course, there is stress induced by genotoxic therapeutics (Bartkova et al. 2006; Gozuacik and Kimchi 2004; Karantza-Wadsworth et al. 2007; Mathew et al. 2007). There is substantial evidence that cells actively upregulate mutagenesis in response to stress by at least two mechanisms: DSB-induced gene amplification, which can increase expression of proteins that confer stress resistance, and switching from accurate to error-prone DSB repair mechanisms. These processes underlie "adaptive mutagenesis" which helps generate mutants that are better adapted to a stressful environment, and in essence, reflect "regulated evolvability" (Galhardo et al. 2007; Gonzalez et al. 2008; Hastings et al. 2009; Ponder et al. 2005). The inherent complexity of DNA repair networks and their regulation, coupled with the immense genetic heterogeneity of solid tumors, presents both challenges and opportunities for developing synthetic lethal and sensitivity approaches to cancer treatment.

2.4 DNA Repair and Genome Instability: Roles in Cancer Etiology, Tumor Progression and Resistance to Therapy

Genome instability was recognized early as a hallmark of cancer. Cancer arises when cells acquire altered cell growth properties including independence from growth signals, immortality, and defects in programmed cell death pathways. Tumor progression to a more aggressive state depends on alterations that affect tissue responses including angiogenesis, tissue invasion, and adaptability to new tissue environments which drive metastasis. Although it has long been known that cancer cells have unstable genomes, it was difficult to establish whether genome instability was an early driver of cancer, or a secondary manifestation of the cancer state. There are now several well-established cases where genome instability has been definitively shown to precede cancer (Hanks et al. 2004; Lengauer et al. 1998; Pikor et al. 2013; Shih et al. 2001; Weaver et al. 2007). Although the number and types of genetic changes required to initiate tumorigenesis and promote tumor progression vary among different types of cancers, a common theme is the early acquisition of defects in DNA repair systems that play critical roles in genome stabilization.

Genome instability manifests over a wide scale, from point mutations, trinucleotide repeat expansions and contractions, gene duplications, deletions, and inversions, to large-scale chromosome changes including translocations and whole chromosome gains and losses. Defects in DNA repair pathways contribute to specific instabilities (Fig. 2.4). Point mutagenesis is greatly increased by defects in BER, NER, and MMR. MMR also suppresses microsatellite repeat expansion or contraction reflecting replication slippage at short repeats such as trinucleotide repeats. cNHEJ and RAD51-dependent HR suppress translocations by aNHEJ and SSA, respectively.

Thus, DNA repair defects cause genome instability, which accelerates the acquisition of mutations in critical growth regulatory genes, including gain-of-function

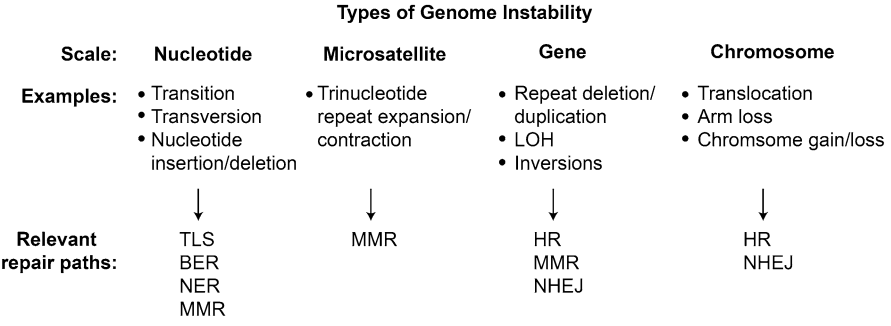

Fig. 2.4 Genome instability and DNA repair. Genome instabilities result in small, moderate, and large scale genome alterations. Indicated repair pathways suppress or induce different types of instabilities

mutations in proto-oncogenes and loss-of-function mutations in tumor suppressor genes (Wang 2005). Defects in specific DNA repair pathways are associated with heritable cancer syndromes such as colon cancer (MMR), skin cancer (NER), and breast cancer (HR) (Boulton 2006; Friedberg 2001; Gudmundsdottir and Ashworth 2006; Jass 2002; Jiricny 2006; Venkitaraman 2002).

Conversion of a normal cell to a metastatic cancer cell may require 3–10 mutations in key cell growth and tissue regulatory genes (Ilyas et al. 1999; Schedin and Elias 2004; Spurgers et al. 2006). Mutation rates in normal mammalian cells are low, ~10^{-10} per base per cell generation, which translates to 10^{-6} to 10^{-8} mutations per gene per cell generation (Baer et al. 2007; Roach et al. 2010). Assuming mutations arise independently, even at the higher rate of 10^{-6} mutations per gene per generation, the odds of accumulating as few as five critical mutations in a single cell is vanishing small (~10^{-32}). DNA repair defects greatly increase mutation rates and the odds of accumulating critical mutations.

Mutations arise infrequently in undamaged DNA, e.g., by base mis-incorporation during DNA replication, but mutation rates are dramatically increased at or near sites of DNA damage as a result of inaccurate DNA repair or error-prone lesion bypass, including translesion DNA synthesis and recombinational mechanisms (Nickoloff 2002; Sale 2013; Shaheen et al. 2011). DNA lesions can arise spontaneously, reflecting the chemical lability of DNA (e.g., deamination of cytosine and 5-methyl cytosine to uracil and thymidine, respectively); damage caused by reactive oxygen species (ROS) formed during normal cell metabolism; and single- and double-strand breaks created by nucleases or when replication forks collapse (Allen et al. 2011; Barnes and Lindahl 2004; Caldecott 2008; Gates 2009). DNA oxidation by ROS is a major source of mutations as it produces many types of lesions including oxidized bases (e.g., 8-oxoguanine), abasic sites, bulky lesions (e.g., etheno and protein-DNA adducts), strand breaks, and DNA crosslinks (Waris and Ahsan 2006). Genotoxic agents are mutagenic because DNA repair does not always accurately restore the original DNA sequence.

When cells acquire a DNA repair defect, increased mutagenesis drives early stage tumorigenesis through alterations in key growth regulatory genes, and it also drives the rapid evolution of tumor cells that promotes tumor progression. As long as mutation rates remain below a critical "error catastrophe" limit (Fox and Loeb 2010), high mutation rates allow tumor cells to "test" mutations that allow them to adapt to various types of stress, including nutrient and oxygen deprivation, a common feature of tumor microenvironments, and to develop resistance to therapy. Most cancer patients are treated with DNA damaging agents, even when tumors are resectable, including chemotherapeutic drugs and/or ionizing radiation. These approaches exploit the fact that tumor cells divide more rapidly than normal cells, and cells actively replicating DNA are highly susceptible to the cytotoxic effects of DNA damage. This is because nearly all types of DNA damage block replicative DNA polymerases, causing forks to stall and eventually collapse to cell-lethal DSBs (Allen et al. 2011; Branzei and Foiani 2010). Nonetheless, tumor cells can be highly resistant to traditional therapies. It seems paradoxical that tumorigenesis can be driven by defects in DNA repair genes yet the resulting rapidly growing cells are

often resistant to DNA damaging agents. Although in certain cases this may reflect the use of genotoxins that create specific types of DNA damage that are repaired by pathways that remain functional in a particular tumor (i.e., failure to appropriately tailor the treatment to the specific DNA repair defect), there are many other ways to resolve this paradox. For example, resistance to therapy may reflect upregulation of other DNA repair pathways or drug efflux pathways, and/or defects in apoptosis and other programmed cell death pathways that are normally triggered by heavy loads of DNA damage. In this light it is noteworthy that ~50 % of tumor cells carry defects in p53, which plays critical roles in apoptosis (Carvajal and Manfredi 2013).

As noted above, the increase in genome instability associated with DNA repair defects promotes the acquisition of mutations, including those that drive changes in other DNA repair, drug efflux, and programmed cell death pathways. The realization that rapid evolution of tumor cells allows them to adapt to stressful environments and acquire resistance to genotoxic therapeutics, has forced cancer biologists to reevaluate therapeutic strategies. The traditional approach to induce DNA damage in tumor cells with chemo- and/or radiotherapy is fairly effective at killing bulk tumor cells, but this damage can also generate (and ultimately select for) a subpopulation of tumor cells that are resistant to therapy, and moreover, potentially generate more aggressive tumor cells leading to local tumor recurrence and progression to a more dangerous, metastatic state. These traditional approaches were initially promising because they provide clear short-term benefits, namely rapid and marked reduction in bulk tumor mass and increased median survival times, but they do not necessarily increase long-term patient survival (Fig. 2.5a). To increase long-term patient survival, i.e., to increase the "tail" of Kaplan-Meier survival curves (Fig. 2.5b), we must shift our focus toward strategies that kill or prevent proliferation of essentially 100 % of tumor cells. The significant difference between median survival time and the fraction of long-term survivors was elegantly explained by Stephen Jay Gould (1985). While the goal of improving long-term survival is clear, achieving this goal presents major challenges given the difficulty in eradicating tumor cells while minimizing the effects of chemo- or radiotherapy

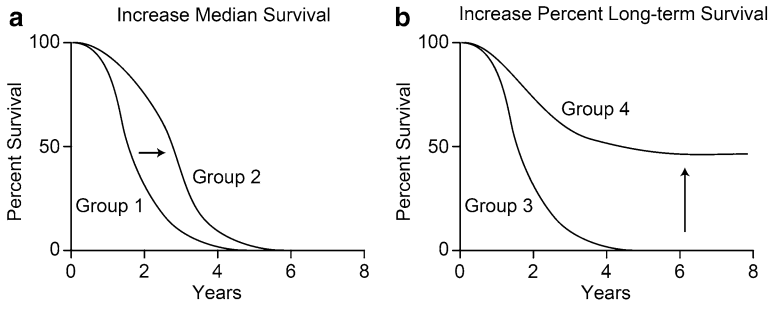

Fig. 2.5 Idealized Kaplan-Meier survival curves showing (**a**) increased median survival time of treatment group 2 vs group 1, but no increase in long-term survival, and (**b**) increased percentage of long-term survivors in treatment group 4 vs group 3

on normal tissue. In addition to causing serious immediate side effects such as neurological and gastrointestinal problems, fatigue, fever, liver and kidney failure, traditional cancer therapies can cause a wide range of serious late effects including cardiac disease, nephrotoxicity, infertility, hearing loss, neurological problems, and secondary tumors (Gururangan 2009). These late effects are a more serious problem for pediatric patients and others with potential for long-term survival. Therapeutic interventions that target tumor weaknesses through synthetic lethal and synthetic sensitivity approaches that exploit known DNA repair, checkpoint, or programmed cell death defects (or target these pathways with inhibitors) could be more effective at eradicating tumor cells while minimizing harm to normal tissues. Because DNA repair plays such a prominent role in tumorigenesis and tumor response to therapy, a deep understanding of DNA repair networks holds significant promise for improving cancer therapy.

2.5 Synthetic Lethality and Sensitivity in Targeted Cancer Therapy

The key to effective cancer treatment is to target tumor cells while sparing normal tissue. Although there have been notable successes identifying tumor-specific targets, such as the brc-abl fusion protein in chronic myelogenous leukemia that can be inhibited with Imatinib (Gleevec) this approach may not be generally applicable to far more genetically heterogeneous solid tumors (Fox et al. 2009). This has led to the idea that the search for cures should focus on "*disrupting the broader biological pathways that support cancer growth*" (Hayden 2008). DNA damaging agents do indeed disrupt a critical biological pathway required for growth (DNA replication), but systemic chemotherapy, and even well-targeted radiotherapy, can cause significant normal tissue damage. Normal tissue tolerance limits the doses that can be delivered to tumors, and increases the chance that some tumor cells will survive. After therapy, surviving tumor cells are likely to have suffered considerable DNA damage which could drive mutagenesis and promote tumor progression upon recurrence. It is therefore imperative to develop targeting strategies that selectively kill tumor cells. Given that DNA repair defects are early drivers of many solid tumors, there is great interest in developing therapeutics that exploit these potential weaknesses based on synthetic lethality and sensitivity. Because unrepaired DSBs are generally lethal to cells, there has been significant focus on DSB repair pathways and genotoxins that directly or indirectly induce DSBs. However, we should not restrict our thinking to just these pathways and agents, as there are many pathways to death or even senescence, which achieve the same goal of preventing tumor growth and spread.

Early *Drosophila* geneticists first defined the concept of synthetic lethality and redundant genetic pathways in terms of genetic compensation for the loss of a required function by dependence on a redundant pathway (Dobzhansky 1946). The concept was formalized for cancer drug discovery by Hartwell and colleagues (1997) which led to an early genetic screen to assess drug sensitivities of yeast with

checkpoint and DNA repair defects (Simon et al. 2000). A recent example of a much larger scale synthetic lethal/sensitivity screen used ~110,000 yeast double knockout mutants in a synthetic genetic array (SGA) approach to identify growth defects and sensitivity to three genotoxins, the alkylating agent methylmethane sulfonate, the radiomimetic zeocin, and the TopI inhibitor camptothecin (Guenole et al. 2013). This study generated ~1,800,000 data points that reveal important DNA repair, checkpoint, and replication interactions clustered into "interaction maps" that suggested novel roles for known proteins including the RTT109 histone acetyltransferase, and roles for previously uncharacterized proteins in DNA repair and checkpoint functions. This and similar datasets will no doubt reveal many new targets to explore for novel synthetic lethal/sensitivity approaches. One of the advantages of the yeast SGA system is the ability to generate large numbers of double-mutant combinations that can be tested in largely unbiased screens. A second advantage (yet to be pursued) is that interesting double-mutants can be backcrossed to large sets of mutants to create triple mutants that can rapidly screened to determine if the double-mutant lethal or drug-sensitive phenotype can be suppressed by a third mutation, and this approach can be iterated to create strains with four or more mutations. Identifying suppressors of the original synthetic lethal or sensitivity phenotype may provide important insight into how cancer cells might evolve resistance to treatments that exploit a particular synthetic interaction, and this might lead to the development of robust protocols that "anticipate" and thereby prevent the development of resistant tumors. Many synthetic lethal or sensitive phenotypes are based on genetic interactions observed with gene inactivating mutations, but the approach is not limited in this way. For example, lethal interactions can arise with gain of function mutations as well, such as cells with activated RAS depending on STK33 and TKB1 kinases for viability (Barbie et al. 2009; Scholl et al. 2009). Yeast again provides a means to efficiently screen for such interactions with available overexpression libraries, offering a means to identify "synthetic dosage lethality" (Jones et al. 2008; Kroll et al. 1996). These approaches may be useful in regard to sensitization of mammalian cells to DSB damage because overexpression of human RAD51 or RAD52 can have dominant negative effects on DSB repair (Kim et al. 2001), and RAD51 is overexpressed in a wide variety of tumor cell lines (Raderschall et al. 2002).

The most dramatic discovery of a synthetic lethal interaction in human cancer was made when the Helleday and Ashworth labs independently tested the hypothesis that HR defects in BRCA1- or BRCA2-defective breast cancers would be synthetically lethal with PARP1 inhibitors (PARP1i) because PARP1 functions in BER, and inhibiting the repair of single-strand damage would increase the frequency of replication fork collapse, and there would be strong requirement for a functional HR system to restart the many collapsed forks (Bryant et al. 2005; Farmer et al. 2005). There was great excitement about these findings for several reasons. BRCA-defective tumor cells were exquisitely sensitive to PARP1i relative to matched BRCA-proficient cells, with differences ranging from ~50- to nearly 1,000-fold, and BRCA-defective tumors were effectively eradicated by PARP1i treatment in mouse models. Thus PARP1i displayed impressive therapeutic gain. Most importantly, the

PARP1i chemotherapy approach with BRCA-defective tumors was unique among cancer therapy strategies that exploit DNA damage sensitivity of tumor cells, in that *no exogenous DNA damaging agents were applied*. Instead, the strategy depended only on the spontaneous damage that is normally present in all cells. Thus, the PARP1i-BRCA interaction is truly synthetically lethal, rather than synthetically sensitive. Nonetheless, for certain tumors (e.g., those resistant to PARP1i alone) it is worthwhile exploring synthetic sensitivity by combining PARP1i with traditional chemotherapeutics. One example of this approach combined the PARP1i AZD2281 with cisplatin and carboplatin, which gave improved treatment outcomes in mouse models (Rottenberg et al. 2008).

The excitement surrounding PARP1i-BRCA synthetic lethal discovery quickly led to clinical trials with several PARP1i candidates; unfortunately, some early trials employed candidates with weak PARP1i activity, which not unexpectedly, gave poor results, but nonetheless slowed the field significantly until the weak compounds were revealed as such (Garber 2013). Additional challenges emerged when it became apparent that BRCA-defective tumors can gain resistance to PARP1i by several mechanisms including loss of PARP1, reactivation of HR, and increased expression of the P-glycoprotein efflux pump (Lord and Ashworth 2013).

Because HR proficient cells are fairly resistant to PARP1 inhibitors, these drugs are well-tolerated by patients, leading to the suggestion that they may be used to prevent cancer in cancer-prone populations including carriers of *BRCA1* or *BRCA2* defective alleles (Vinayak and Ford 2010). There are a wide range of human tumors with known or suspected defects in HR. The best studied of these have defects in BRCA1 or BRCA2, but defects in many other HR factors are known or suspected to occur in cancers including ATM, ATR, each member of the MRN complex, RAD51, RAD51 paralogs XRCC2 and XRCC3, and members of the Fanconi anemia family (FANCF, FANCJ, FANCC, FANCA, and FANCG). Tumors harboring these HR defects include women's cancers (breast, ovarian, endometrial, and cervical cancer), men's cancers (prostate, male breast cancer) and others including pancreatic, head and neck, brain, thyroid, lung, gastrointestinal, and melanoma (Cerbinskaite et al. 2012). Interestingly, HR defects have also been found in blood tumors including leukemia, multiple myeloma, and lymphoma (Cerbinskaite et al. 2012). Thus, PARP1i could have broad applicability for treating tumors that exhibit "BRCAness" (Bast and Mills 2010; Turner et al. 2004). If a tumor doesn't exhibit BRCAness, this state can be induced by inhibiting HR, for example with proteasome or HSP90 inhibitors, or siRNA downregulation of BRCA2 (Gudmundsdottir et al. 2007; Noguchi et al. 2006; Yu et al. 2008).

Genetic screens and other approaches continue to identify novel synthetic lethal and synthetic sensitive interactions. siRNA screens in mammalian cells have identified additional gene targets that when repressed result in synthetic lethality or sensitivity to PARP1i. Such screens identify HR factors including BRCA1 and BRCA2, as expected, but interestingly, genes that operate in DNA repair and other pathways are also recovered, i.e., NER proteins DDB1 and XAB2, and the PI3K regulator PTEN (Lord et al. 2008; Mendes-Pereira et al. 2009). PARP1i are also synthetically lethal with ERCC1 defects associated with lung cancer (Postel-Vinay

et al. 2013). Many other DNA repair-based synthetic lethal interactions have been found, such BRCA1 and Tankyrase 1, MMR proteins MSH2 or MLH1 with DNA polymerases POLB and POLG, ATM and p53, and ATR and p53 (Jiang et al. 2009; Martin et al. 2010; McCabe et al. 2009; (Nghiem et al. 2001). These and other examples point to the rich opportunities that lay ahead in the search for more effective, targeted cancer therapies.

2.6 Concluding Remarks

The exploration of DNA repair pathways to develop new synthetic lethal/sensitivity approaches has great potential for identifying novel targeted cancer therapies. Naturally, these investigations have largely taken a genetic approach, using gene knockouts, gene knockdowns, and chemical inhibitors. It is important to remember that there are many ways to target cancer and thereby increase therapeutic gain. For example, radiotherapy provides a physical approach to targeting tumors and improvements in beam focusing and "dose painting" continue to increase the ratio of dose delivered to tumor volumes relative to surrounding normal tissue. Localized drug treatment to sensitize tumors to radiation could provide significant advantages, especially if such drugs were themselves targeting tumor-specific synthetic lethal interactions. An interesting example of this type of approach is based on the observation that the complex DNA damage caused by carbon ion radiation is poorly repaired by NHEJ, thus tumor cells rely on HR to repair this damage (Okayasu et al. 2006). In effect, carbon ion radiation damage mimics an "NHEJ-defective" state, and when HR is inhibited, either by targeting BRCA2 with siRNA, or by downregulating RAD51 with HSP90 inhibitors, carbon ion radiotherapy efficacy is substantially increased (Noguchi et al. 2006; Yu et al. 2008). This approach is analogous to familiar synthetic lethal approaches as it restricts one pathway (NHEJ) for repair, and then targets the remaining pathway (HR). Because cancer cells are highly proficient at adapting to stress, combining, or "layering" multiple targeted approaches may offer the best opportunities to enhance therapeutic gain and prevent rare surviving tumor cells from developing resistance to subsequent therapy (Kon et al. 2012; Nickoloff 2013).

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Chapter 3
Cell Cycle Checkpoint and DNA Damage Response Defects as Anticancer Targets: From Molecular Mechanisms to Therapeutic Opportunities

Loredana Spoerri, Zay Yar Oo, Jill E. Larsen, Nikolas K. Haass, Brian Gabrielli, and Sandra Pavey

Abstract Cells have evolved a DNA damage response (DDR) pathway to monitor the integrity of their genome, which is tightly associated with cell cycle checkpoint controls, arresting cells to allow for DNA repair before continuing through the cell cycle. Defects in the DDR and checkpoint mechanisms frequently occur in human cancers, with failure of the cell to repair the DNA damage leading to genomic instability, increased mutation load, and cellular transformation. The loss of a DNA damage checkpoint in a tumour should make it vulnerable to checkpoint override strategies, providing therapeutic opportunities to inhibit mechanisms that compensate for the defect. Here we review the DDR pathway and cell cycle checkpoint responses to DNA damage, and explain how defects in these mechanisms present a significant opportunity for therapeutic intervention. These defects can be exploited using a synthetic lethal approach to target tumours with these defects and having limited normal tissue toxicity.

Keywords Cell cycle • Mitosis • Checkpoints • DNA damage • DNA damage response • DNA repair • Cancer • Chemotherapy • Radiotherapy • Synthetic lethality

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Loredana Spoerri and Zay Yar Oo contributed equally.

L. Spoerri (✉) • Z.Y. Oo (✉) • N.K. Haass • B. Gabrielli • S. Pavey
The University of Queensland Diamantina Institute, Translational Research Institute, The University of Queensland, Level 6 West, 37 Kent Street, Woolloongabba, Brisbane, QLD 4102, Australia
e-mail: l.spoerri@uq.edu.au; z.oo@uq.edu.au; n.haass1@uq.edu.au; briang@uq.edu.au; sandra.pavey@uq.edu.au

J.E. Larsen
QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Brisbane, QLD 4006, Australia
e-mail: Jill.Larsen@qimrberghofer.edu.au

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3.1 Introduction

The advent of targeted therapies based on knowledge of mechanisms that drive cancer cell proliferation, inhibit apoptosis or evasion from the immune system has revolutionised cancer therapy. However, despite unprecedented responses to some small-molecule inhibitors or monoclonal antibodies, these approaches are curative in only a subset of patients. One reason for this is the often rapid development of acquired drug resistance. Therefore, new approaches are urgently needed.

Hanahan and Weinberg have identified the ten hallmarks of cancer, dysregulated mechanisms or defects required for cancer initiation, survival and metastasis (Hanahan and Weinberg 2000, 2011; Hanahan 2014). All these hallmarks are also useful therapeutic targets as they differentiate cancer from normal tissue, and the cancer is dependent on these changes for survival. One of these hallmarks, namely 'genome instability and mutation', has been used as an unselective target for decades, with many of the conventional chemotherapeutic agents increasing DNA damage to effectively overloading defective repair and response mechanisms to trigger cancer cell death. However, our improved understanding of the various DNA damage responses now allows us to more effectively target specific defects to deliver a tumour selective cytotoxic insult with minimal or at least much reduced normal tissue toxicity. In this chapter we review the concept of defects in DNA damage response and their related cell cycle checkpoints as anticancer targets.

3.2 Cell Cycle and Cell Cycle Checkpoints

3.2.1 Cell Cycle

The cell cycle is a sequence of cellular processes that regulate cell division. It consists of four phases, two gap phases G1 and G2, where the cell grows, S phase where DNA replication occurs, and the M phase (mitosis and cytokinesis) where the cell divides the duplicated genome into two identical daughter cells. During M phase, chromatin condenses into chromosomes (prophase) which become attached to the spindle microtubules (prometaphase). Chromosomes then align at the midline of the cell (metaphase), and the duplicated chromosomes are separated into sister chromatids (anaphase). Cytokinesis, the actual division into the daughter cells, is the final step of M-phase. The sequential progression of the cell cycle phases is coordinated by the activity of cyclin-dependent kinase proteins (CDKs) which are in turn regulated via the binding to cyclins (Fig. 3.1), a family of proteins whose members are individually synthesised and degraded in a phase specific manner.

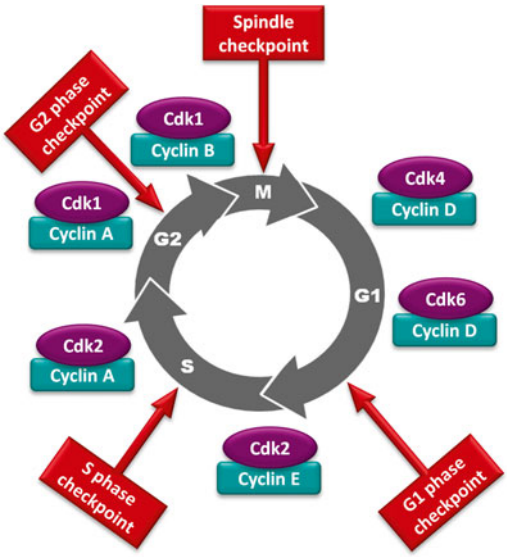

Fig. 3.1 The cell cycle and its checkpoints. Cyclins are individually synthesised and degraded in a phase distinctive manner and bind and activate specific Cdks which coordinates the sequential progression of the cell cycle phases. Four main checkpoints, one in each cell cycle phase, respond to different types of DNA insults and promote cell cycle arrest and DNA damage repair

3.2.2 Cell Cycle Checkpoints

The cell division cycle is surveyed by multiple checkpoints that respond to a wide range of internal and external stresses to ensure genomic integrity from one cell generation to the next. When checkpoints are triggered, CDKs are generally inhibited (the exception being the spindle assembly checkpoint in mitosis where inactivation of the CDK is inhibited); this results in cell cycle arrest and provides time for DNA repair to occur. If the damage is extensive and cannot be resolved, checkpoints trigger cell senescence or apoptosis.

There are four main checkpoints responding to different types of DNA insults, one in each cell cycle phase (Fig. 3.1). The G1 phase checkpoint prevents the replication of damaged genomic material by blocking entry into S phase, the S phase checkpoint not only responds to the presence of DNA damage but also to aberrant replication forks by stopping or slowing DNA synthesis, the G2 phase checkpoint impedes cells with damaged or entangled, catenated DNA from undergoing mitosis, and finally the spindle assembly checkpoint only allows mitotic exit if the chromosomes are properly attached to the mitotic spindle.

3.2.3 Cell Cycle Signalling Pathways

Two major players in checkpoint signalling in response to DNA damage are the ataxia telangiectasia mutated (ATM) and the ataxia telangiectasia and Rad3-related protein (ATR) proteins. ATM is mainly triggered by double strand DNA breaks (DSBs),

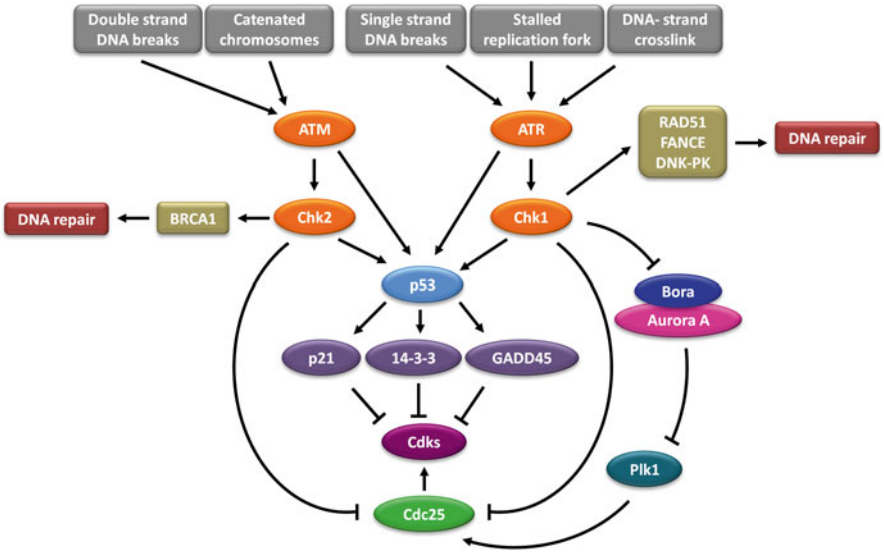

Fig. 3.2 Cell cycle checkpoints signalling pathways. In response to different types of DNA damage, ATM and ATR are activated and subsequently upregulate Chk1 and Chk2. In turn, Chk1 and Chk2 inhibit Cdks by deactivating Cdc25 and by promoting the transcription of p21, 14-3-3 and GADD45 Cdks inhibitory factors via p53 activity. Differently from all the others, the spindle assembly checkpoint maintains Cdk1/Cyclin B activity which in turn hampers mitotic progression. Beyond promoting a cell cycle checkpoint arrest, Chk1 and Chk2 also activate the DNA damage repair mediated through RAD51, FANCE, DNK-PK and BRCA1 proteins

while ATR primarily responds to single-stranded DNA (ssDNA) (Fig. 3.2). Once activated, these kinases phosphorylate and activate checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) proteins, which in turn target a range of effectors that inhibit cell cycle progression, including the immediate activators of the CDK/Cyclins, the Cdc25 phosphatases. ATM/ATR and Chk2/Chk1 also phosphorylate and activate p53 protein which promotes the transcription of p21, GADD45 and 14-3-3 CDK inhibitor proteins (Elledge 1996; Ciocca and Elledge 2000; Elledge et al. 2000; Huang and Elledge 2000; Liu et al. 2000; Schulman et al. 2000; Tibbetts et al. 2000; Wang et al. 2000; Zhou and Elledge 2000), thus enhancing cell cycle arrest. Another mitotic regulatory pathway involves Plk1 protein kinase which is activated by the cooperative action of Aurora A protein kinase and its cofactor Bora (Seki et al. 2008; Takaki et al. 2008). Plk1 activity is also regulated by ATM/ATR checkpoint signalling (Smits et al. 2000; van Vugt et al. 2001). A link between this pathway and the G2 phase DNA damage checkpoint has been recently described where Bora is degraded via ATR-mediated phosphorylation following DNA damage (Qin et al. 2013), and ATM-dependent phosphorylation and activation of B56 regulatory subunit of PP2A that is responsible for dephosphorylating and inactivating Plk1 (Shouse et al. 2011).

In response to DNA lesions, cells initiate a highly coordinated cascade of events, known as the DNA damage response (DDR), which is essential for the maintenance

of genomic stability and cell survival. DNA damage can interfere with essential cellular processes, such as transcription or replication, and can compromise the viability of the cells. DNA damage repair is tightly coordinated with cell cycle progression through the activation of DNA damage checkpoints. The DDR consists of detection of damage by sensor proteins, with signal transducer and effector proteins launching a cascade of events that causes cell cycle arrest, activation of DNA repair, senescence or apoptosis.

DNA damage is detected by sensor proteins, with DSBs detected by the MRE11-RAD50-NBS1 (MRN) sensor complex and ssDNA arising from DNA damage processing is signalled by Replication protein A (RPA), an ssDNA binding protein. Once DNA damage is sensed, the cell must transduce this signal down to its appropriate effectors. The MRN complex leads to the recruitment and activation of ATM and RPA recruits the ATR kinase via its partner protein, ATRIP (ATR-interacting partner) (reviewed in Elledge 1996; Zhou and Elledge 2000). ATM and ATR are related kinases that phosphorylate a number of mediators which are mostly cell cycle specific and associate with damage sensors, signal transducers and effectors at particular phases of the cell cycle, and as a consequence, provide signal transduction specificity. ATM and ATR are both members of the PI-3K-like kinase family (PIKK) of protein kinases that also includes DNA dependent protein kinase (DNA-PK) and mammalian target of rapamaycin (mTOR). The ATM signalling cascade includes 53BP1, MDC1, BRCA1, MCPH1, and PTIP, while mediators of ATR signalling include TopBP1, and Claspin (reviewed in Marechal and Zou 2013). The transducer kinases lead to the activation of the effector kinases CHK1 and CHK2, activating signalling cascades with downstream targets including transcription factors, cell cycle regulators, apoptosis and DNA repair factors (Fig. 3.2). There is crosstalk between the ATM/Chk2 and ATR/Chk1 pathways and they share many substrates.

3.3 DNA Damage Response and Repair Mechanisms

It is estimated that each of the ~10^{13} cells within the human body incurs tens of thousands of DNA-damaging events per day (Lindahl and Barnes 2000). To avoid the deleterious consequences of damage accumulation, multiple DNA repair pathways have evolved, each associated with specific classes of lesions.

DNA is subject to a high level of endogenous damage resulting from reactive oxygen species (ROS) generated from normal cellular metabolism, in addition to a number of other factors including spontaneous hydrolysis, abasic sites, and alkylation. Endogenous sources of damage can arise from physiological DNA processing and DNA repair processes themselves, such as DNA mismatches, insertions and deletions which can be introduced as a result of misincorporation of bases by replicative DNA polymerases (McCulloch and Kunkel 2008).

There are a number of exogenous or environmental sources of DNA-damaging agents including ultraviolet radiation (UV), ionizing radiation (IR), chemical agents used as clinical and chemotherapeutic drugs, tobacco smoke and other environmental agents. Environmental stresses such as ultraviolet light (UV) from the sun, primarily causes two types of DNA lesions, cyclobutane pyrimidine dimers (CPDs) and 6–4

pyrimidine products (6–4PP) (You et al. 2001). IR is the most common DSB-inducing agent, and can originate from both natural (gamma and cosmic radiation) and artificial sources (eg. X-rays and radiotherapy). IR can also induce DNA damage indirectly through the production of ROS.

Chemical agents used as chemotherapeutic drugs, including alkylating agents (eg. temozolomide), bifunctional alkylating agents (e.g. platinum agents), and mitomycin C cause DNA damage in the form of intra-strand and inter-strand cross-links. Targeted drugs such as topoisomerase I or II inhibitors, generate ssDNA or dsDNA breaks by trapping topoisomerase-DNA covalent complexes (Sinha 1995). Nitrosamines are a class of potential carcinogens that are found in tobacco smoke, and there is a possible dose-response relationship between the amount and duration of tobacco smoke exposure and mutational burden (Govindan et al. 2012). Other naturally occurring environmental agents include N-nitrosoamines, heterocyclic amines, and polycyclic aromatic hydrocarbons which are common in the diet, producing bulky DNA adducts (reviewed in Dexheimer 2013).

3.3.1 Types of DNA Damage Repair

DNA damage repair can be subdivided into several distinct mechanisms based on the type of DNA lesion (Fig. 3.3), summarised below.

3.3.1.1 Nucleotide Excision Repair (NER)

NER is the major repair pathway for removal of bulky DNA adducts which distort the normal structure of the DNA helix. These can be formed by ultraviolet radiation (yielding adducts like thymidine dimers or 6–4 photoproducts), chemicals or ROS, which produce lesions that disrupt transcription and replication. NER also contributes to the repair of intra- and interstrand crosslinks (ICLs). Two NER sub-pathways exist: global genome NER (GG-NER) which repairs damage that occurs throughout the entire genome, while transcription-coupled repair (TC-NER) preferentially repairs damage in transcriptionally active genes (reviewed in Naegeli 1995). Both pathways converge into a common pathway involving over 20 different genes. Hereditary defects in NER cause UV sensitivity and skin cancer development (Andressoo et al. 2005). There are three separate though inter-related autosomal recessive disorders including Xeroderma pigmentosum, Cockayne Syndrome and Trichothiodystrophy (TTD). Subjects with Xeroderma pigmentosum exhibit >100-fold increased incidence of sun-induced skin cancer (Arlett and Lehmann 1996).

3.3.1.2 Base Excision Repair (BER)

BER is involved in the removal of a variety of endogenous and exogenous DNA lesions, repairing mostly alkylated, deaminated and oxidized bases, in addition to abasic sites. BER is a multistep process that requires the sequential activity of more

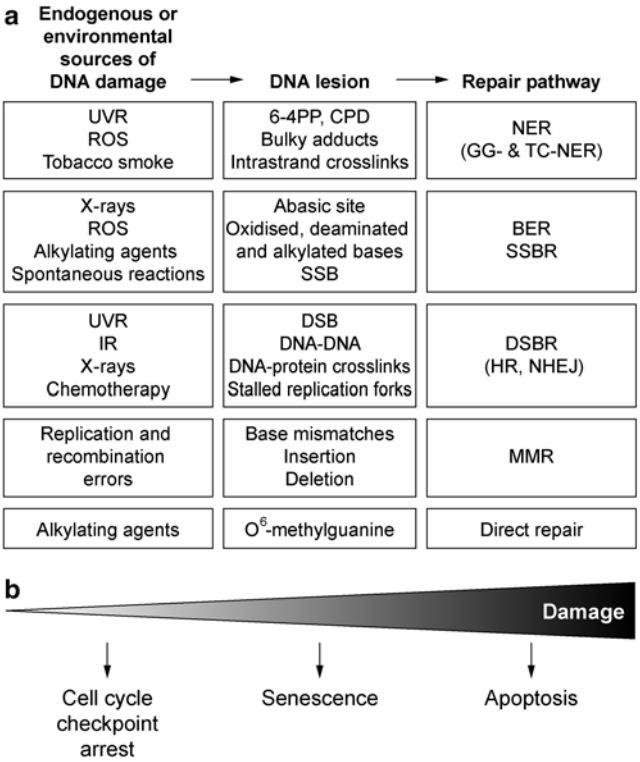

Fig. 3.3 DNA damage repair. (**a**) Sources of DNA damage and the specific mechanisms used to repair the DNA lesions. (**b**) The extent of DNA damage and success of repair determine whether the cell undergoes cell cycle checkpoint arrest to allow for repair, or where damage is too extensive the cell encounters senescence, or death

than 20 proteins and consists of several distinct BER sub-pathways, dependent on the type of DNA damage encountered, with different glycosylases detecting different types of base damage (Dizdaroglu 2005). Mutations and polymorphisms in BER genes have been associated with aging, cancer susceptibility and neurodegeneration (Dianov and Hubscher 2013).

Single Strand Break Repair (SSBR)

Single strand breaks (SSBs) in DNA are the most common endogenous lesions, arising both directly from DNA damage induced from ROS and indirectly from BER (reviewed in Caldecott 2008). Whilst SSBs are not as harmful as DSBs, they can block DNA replication and transcription, and occur three orders of magnitude more frequently than DSBs (Bradley and Kohn 1979). SSBs can arise as BER intermediates, there are also numerous involuntary ssDNA breaks that can also occur through direct and indirect mechanisms. Exogenous sources of SSBs include IR

and UV radiation that can generate SSBs during lesion repair, or can be the result of genotoxic agents such as chemotherapeutic drugs. The most likely consequence of unrepaired SSBs is the blockage or collapse of DNA replication forks during S phase, possibly leading to the formation of DSBs.

Repair of SSBs occurs by a GG-NER pathway throughout the genome and throughout the cell cycle to rapidly detect and remove the majority of chromosomal SSBs (reviewed in de Laat et al. 1999). However, SSBs encountered by the DNA replication machinery during S-phase can cause the replication fork to stall, risking replication fork collapse. Cells have developed a DNA-damage tolerance pathway known as post-replication repair, which enables the cells to complete bulk DNA replication and remove the lesions after replication is completed (Wigan et al. 2012). This may be undertaken using one of two mechanisms, an error-free recombination repair mechanism, and an error-prone translesion synthesis (TLS) mechanism. Defects in SSB repair cause neurological disorders including Ataxia-oculomotor apraxia 1 (AOA1), and Spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) (Rass et al. 2007).

Double Strand Break Repair (DSBR)

DNA DSBs are the most deleterious form of DNA damage because they do not leave an intact complementary strand to be used as a template for DNA repair. If left unrepaired, they can ultimately lead to chromosome breaks and translocations (Jackson and Bartek 2009). Hence repair of DSBs is both critical for cell survival and maintenance of genome integrity. DSBs can be generated in response to IR, many chemotherapeutics (etoposide, doxorubicin, camptothecin derivatives) which act as topoisomerase poisons, from replication of DNA containing SSBs, and naturally occurring DSBs arising at chromosome ends when telomeres become critically short during replicative senescence. DSB repair utilises two major pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). These pathways are complementary and operate optimally under different circumstances.

Homologous recombination is a highly complex process that involves multiple proteins and occurs during late S and G2 phases of the cell cycle, requiring the presence of a homologous template (for review see Li and Heyer 2008). The broken DNA ends of a DSB are resected to allow invasion of the single strands into the sister chromatid, which functions as a template for accurate resynthesis of the damaged DNA. This pathway repairs only a minor proportion of DSBs, however it may be the most crucial as it is high fidelity, and it deals with stalled and collapsed replication forks, as well as single-ended DSBs, and also ICLs. HR is crucial for the maintenance of genomic stability, and the function of the entire pathway can be compromised by mutation in one or more genes. Many tumour suppressor genes participate in this pathway including *BRCA1* and *BRCA2* which are mutated in hereditary breast cancers, and *ATM*.

NHEJ repairs double-stranded DNA breaks without the need for template DNA. NHEJ is active in all phases of the cell cycle, predominating in G1 phase, and

mediates the direct ligation of broken DNA ends, with minimal DNA end processing. NHEJ is the prevalent DSB repair pathway, however it has the potential to be error prone, as deletions or insertions can be induced at sites of repair (Lieber et al. 2010). While there have been reports of some single nucleotide polymorphisms in NHEJ associated gene loci linked to cancer and epigenetic silencing of NHEJ components, defects in members of the NHEJ pathway have infrequently been identified in association with cancer. Cells that lack any of the NHEJ components are radiation sensitive.

3.3.1.3 Mismatch Repair (MMR)

The MMR pathway repairs errors that have occurred during normal DNA replication caused by the incorporation of the wrong nucleotide, nucleotide deletions or insertions, and have escaped the proofreading activity of replicative polymerases. MMR is also required for the removal of bases damaged by methylating agents and antimetabolites and possibly intrastrand crosslinking agents (chemotherapy). There are essentially three steps in the repair process: a recognition step where mispaired bases are recognized, an excision step where the error-containing strand is degraded resulting in a gap, and a repair synthesis step, where the gap is filled by the DNA resynthesis (for review of MMR, see Stojic et al. 2004).

Defects in MMR leads to a mutator phenotype which causes cancer predisposition and also affects DNA damage signaling, recombination, and several other DNA metabolic processes (Jiricny 2006). Mutations in MMR genes are associated with hereditary non-polyposis colon cancer (HNPCC), with MMR defective cells unable to correct errors caused by DNA polymerase slippage at repetitive sequences in the genome (for review see Cleaver et al. 2009).

3.3.1.4 Oxidative Damage Repair

O^6-methylguanine-DNA methyltransferase (MGMT) demethylates O^6-methylguanine lesions, which are formed as a result of erroneous methylation and other alkylations induced by dietary nitrosamines or chemotherapy agents. Unrepaired O^6-methylguanine is mutagenic because distorted pairing with cytosine or thymidine leads to G:C to A:T transitions on replication (Saffhill et al. 1985).

3.4 Defective Cell Cycle Checkpoints and DNA Damage Repair in Cancer

Defective cell cycle checkpoint function may increase spontaneous and induced gene mutations and chromosomal aberrations by reducing the efficiency of DNA repair. Cell cycle checkpoints integrate DNA repair with cell cycle progression

and are commonly deregulated in cancers leading to increased genomic instability. This provides cancer cells with an evolutionary or adaptive advantage, allowing them to modify their transcriptome and/or genome to increase their ability to thrive in new tissue environments.

Cell cycle checkpoint pathways integrate repair of specific DNA lesions with the cell cycle position. The phase of the cell cycle in which a checkpoint is induced, and the duration of the arrest is dependent on the type of triggering DNA lesion, the number of lesions and the length of time required to repair the lesions (for review see Lazzaro et al. 2009). There are some lesions that are rapidly repaired and do not require cell cycle checkpoint arrest, while in some cases the cells may keep cycling and reach a cell cycle phase where the specific damage is less toxic or more easily repaired.

G1/S phase transition is a major target of alterations in cancer. Such alterations often result in changing the Retinoblastoma protein (Rb) phosphorylation/dephosphorylation balance and consequently effecting the cell proliferation and blocking entry into S phase (Hall and Peters 1996). Rb point mutations or deletions are found in a wide variety of human cancers, particularly retinoblastomas and sarcomas. Histone deacetylase (HDAC)-Rb complex formation is also inhibited by HDAC point mutations found in human cancer (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998).

P16-mediated senescence is another G1 phase mechanism that responds to extensive DNA damage (Robles and Adami 1998; Shapiro 2006). CDKN2A is the gene which encodes the cell cycle inhibitor $p16^{INK4A}$, which is commonly defective in melanoma (Bartkova et al. 1996; Castellano et al. 1997; Hayward 2003). P16, a major driver of senescence, creates G1 phase arrest by blocking Rb inactivation through CDK4/6-Cyclin D inhibition. Melanoma associated mutation of p16 disrupts its ability to promote senescence arrest (Haferkamp et al. 2008). In addition to this role, increased p16 expression has been correlated with the G2 phase checkpoint arrest in response to suberythemal UVR (Pavey et al. 1999, 2001; Abd Elmageed et al. 2009).

The tumour suppressor p53 is involved in DNA damage response and is inactivated in 50 % of all human cancers (Hollstein et al. 1991), and humans carrying a germ-line deletion of one *TP53* allele are highly prone to cancer development (Malkin et al. 1990; Srivastava et al. 1990). In the face of a range of genotoxic insults, p53 is rapidly stabilized and its increased level promotes cell cycle arrests in G1 and G2 phases in cell lines *in vitro* and signals apoptosis in response to excessive damage. The G1 checkpoint is activated by ATM signalling to Chk2 and p53, among other targets, preventing the replication of damaged DNA by blocking entry into S-phase. ATM promotes Homologous recombination repair by recruiting BRCA1 to DSBs, but can also antagonize BRCA1 and promote NHEJ by recruiting 53BP1. Defects in the function of p53 may contribute to the genetic instability that appears to drive neoplastic evolution.

The decatenation checkpoint is another G2 phase checkpoint that has been reported to be defective in a large proportion (68 %) of melanoma cell-lines (Brooks et al. 2014). This ATM/Chk2-dependent checkpoint ensures that chromosome catenation, normally resulting from DNA replication, is resolved before the cell progresses into mitosis. The difference in checkpoint functional response was not

attributable to Topoisomerase II levels. Intriguingly, despite the absence of cycle arrest, the checkpoint defective cell-lines were able to activate the checkpoint signalling following treatment with the topoisomerase II inhibitor ICRF-193, indicating participation of other players, possibly involved in checkpoint recovery, in this phenomenon (Brooks et al. 2014).

The mitotic spindle assembly checkpoint (SAC) monitors spindle defects. Many of the spindle assembly checkpoint sensors and effectors are localized to kinetochores, including the Mad and Bub gene families and Aurora kinases. This checkpoint detects defects in spindle attachment to the replicated chromosomes through the centromere localised kinetochore complex, and tension across the centromere, to ensure the fidelity of partitioning of the replicated genome into the daughter cells. Although mutations in SAC genes are uncommon, they have been reported at low frequency in a number of cancer types (Cahill et al. 1998). The microtubule direct drugs, taxane, vincalkaloids, and some of the newer targeted drugs such as Aurora inhibitors target this checkpoint. Cancer cells appear less able to maintain the mitotic arrest in the face of these drugs resulting in mitotic slippage and triggering cell death (Dowling et al. 2005; Weaver and Cleveland 2005; Gabrielli et al. 2007; Stevens et al. 2008).

3.5 Exploiting Defective Cell Cycle Checkpoints and DNA Damage Response in Cancer Therapy

Increased endogenous damage levels in cancer cells caused by defects in the cell cycle checkpoints and deregulation of DNA repair mechanisms can lead to genomic instability and contribute to the initiation and progression of cancer. This must be accommodated by the cells employing either novel mechanisms to cope with the stress, an adaptation to accommodate the stress, becoming more reliant on alternative stress response mechanisms or a combination of all three. Up-regulated DDR pathways can confer therapy resistance to DNA-damaging chemotherapy and radiotherapy, while down-regulated DDR pathways can lead to dependence on a compensatory pathway. This increased reliance on alternative mechanisms to cope with a stress makes it plausible to disarm these mechanisms to selectively target the cancer cells population while normal cells are being protected by their intact checkpoint and repair responses.

3.5.1 Chemotherapy

Most of the conventional chemotherapeutic drugs target rapidly proliferating cell populations by creating high levels of DNA damage and causing cell cycle arrest and cell death. Despite their broad clinical applications, there are serious drawbacks to the use of DNA damaging agents such as a narrow therapeutic index due to poor

selectivity for cancer cells compared to normal proliferating cells, and a frequent loss of effectiveness due to innate or acquired resistance to chemotherapy. Resistance to the treatment can emerge from multiple mechanisms including drug uptake and metabolism, alterations in DNA repair pathways and defects in apoptotic mechanisms (Fodale et al. 2011). Cells respond to DNA damage and/or aberrant replication stress by activating cell cycle checkpoints, slowing cell cycle progression in order to facilitate DNA repair or promoting cell death with irreparable DNA lesions (Langerak and Russell 2011). As a result, sensitivity to these chemotherapeutic agents is reduced by normal checkpoint function.

3.5.1.1 Alkylating Agents

Alkylating agents are one of the common types of chemotherapeutic drugs that prevent the cancer cells from proliferating by creating intra- and interstrand cross-links in DNA. The alkylating agents are not phase-specific and work in all phases of the cell cycle. Depending on the p53 status, they will cause G1, S and G2 phase arrests in cancer cells. In p53 wild type cells, p53 dependent activation of DNA damage repair components will promote repair and result in diminished activity of alkylating agents (Barckhausen et al. 2014). For instance, Dacarbazine (5-(3,3-Dimethyl-1-triazenyl) imidazole-4-carboxamide, DTIC) only displays 10–20 % response rates with no demonstrated impact on overall survival in melanoma patients (Chapman et al. 1999; Wagner et al. 2000), while temozolomide, an orally available imidazotetrazine derivative of dacarbazine, also demonstrates the similar pattern (Middleton et al. 2000). Defects in Mismatch Repair (MMR) cause tolerance to temozolmide, platinum agents and some nucleoside analogues, leading to drug resistance, with adverse reactions observed in clinical trials (reviewed in Kinsella 2009).

3.5.1.2 Antimetabolites

Antimetabolites, such as 5-FU and gemcitabine, deter DNA synthesis of cancer cells by inhibiting either thymidylate synthase or ribonucleotide reductase and, in addition, they disrupt strand synthesis by incorporating into DNA (Iwasaki et al. 1997; Longley et al. 2003). Thus, these antimetabolites can trigger an S phase arrest and reduce the efficacy of these drugs through the normal checkpoint role of blocking cell cycle progression and facilitating repair of the drug induced damage. For example, as high as 50 % of metastatic colorectal cancer patients are insensitive to 5-FU-based chemotherapy (Douillard et al. 2000; Giacchetti et al. 2000; Alhopuro et al. 2005). Gemcitabine, which is now considered as the standard treatment, has a response rate of less than 20 %, although it does provide an improvement in the quality of life in pancreatic cancer patients (Burris et al. 1997).

Genotoxic therapies trigger ATM-/ATR-dependent checkpoint arrest and the checkpoint response defects dependent on these signalling pathways indicate that

differentiating patients on the basis of checkpoint signalling or repair defects in their tumours could increase the response and survival rates. For instance, in breast cancer patients, BRCA mutant patients defective for homologous recombination repair of the cisplatin induced DNA damage responded better to cisplatin treatment (Silver et al. 2010; Curtin 2012). Defects in NER have been used to confer sensitivity to platinum based chemotherapeutics, which reflects a reduced capacity to repair ICLs (Usanova et al. 2010). Alterations in the XPC gene (ERCC5) expression through loss of heterozygosity (LOH) and polymorphisms have been shown to be prognostic indicators to platinum based chemotherapy in lung, ovarian and colon cancers.

Higher levels of DNA repair enzyme MGMT are frequently observed in tumour tissue, and pseudo-substrates have been shown to deplete MGMT, with increased nitrosourea and temozolomide cytotoxicity, but they have been limited by toxicities in normal tissues (Ona et al. 2009; Tubbs et al. 2009). In glioma, MGMT activity is reduced due to promoter methylation, and renders tumour more sensitive to temozolomide and radiotherapy treatment (Hegi et al. 2005).

3.5.2 Radiotherapy

A number of members of the BER pathway have been used as targets for the modulation of radiosensitivity, including Pol β, FEN1, ligase 1, ligase 3, APE1, and PARP inhibitors. Tumours with defects in HR are highly sensitive to crosslinking agents and DSBs that are induced by chemotherapeutic agents and IR. Genes of the HR pathway, including BRCA1, BRCA2 and ATM, are found to be deregulated in hereditary and sporadic tumours, and these defects can be exploited through an increased sensitivity to agents inducing DNA damage repaired by the HR pathways, such as radiotherapy.

Abrogation of cell cycle arrest through inhibition of cell cycle checkpoint signalling molecules such as Chk1 and Chk2 has proved to sensitize various cancer cells to ionizing radiation (Ree et al. 2004; Tao et al. 2009; Morgan et al. 2010). Based on the findings that many cancer cells have a defective p53-dependent G1 checkpoint conferring a dependence on the G2 checkpoint, abrogation of G2 cell cycle checkpoint has been envisaged as a cancer cell specific therapy (Kawabe 2004).

3.5.3 Targeting Defective DNA Repair and Checkpoint Responses

3.5.3.1 CDK Inhibitors

The defective DNA damage and mitotic checkpoints often lead to misregulated CDK activity, and inappropriate tumour cell cycle progression in the presence of unrepair DNA damage can result in genomic instability. There are a number of cell

cycle inhibitors that are undergoing human clinical trials, with most of the reported CDK inhibitors being trialled in solid tumours or leukaemia (Cicenas and Valius 2011). Flavopiridol (Alvocidib) and Roscovitine (Seliciclib, CYC202) were the first two CDK inhibitor compounds to enter clinical trials, with Flavopiridol being evaluated in chronic lymphocytic leukaemia (Ramaswamy et al. 2012). Although their single-agent activities may be modest, CDK inhibitors improved cytotoxic efficacy and overcame drug resistance through combination with chemotherapy (Lapenna and Giordano 2009). The combination of flavopiridol, fludarabine and rituximab (FFR) was studied in a Phase I trial in patients with mantle-cell lymphoma, chronic lymphocytic leukaemia or indolent B-cell non-Hodgkin's lymphoma, producing an 80 % response rate in patients with MCL (Lin et al. 2010). Another example is SNS-032 which selectively inhibits CDK1,2,7 and 9 and results in G2 and M phase cell cycle arrest and apoptosis (Chen et al. 2010; Tong et al. 2010). Even though it showed good preclinical activity, it was discontinued due to high toxicity *in vivo*.

3.5.3.2 Checkpoint Inhibitors

The G2 phase checkpoint response reduces the efficacy of conventional chemotherapeutic agents in p53 defective cancers incapable of a G1 phase arrest, suggesting inhibition of the G2 phase checkpoint to increase sensitivity. Chk1 is a common component of the G2 phase checkpoint in response to a range of chemotherapeutics, and the older, relatively non-specific inhibitors of Chk1 demonstrated that inhibition of Chk1 in combination with chemotherapeutic agents offered an opportunity to potentiate chemotherapy responses in p53 mutant cancers. More recently developed, potent, selective Chk1 inhibitors have demonstrated single agent activity *in vitro*, targeting cells with high levels of replicative stress, although at present there is little *in vivo* evidence to support effective single agent targeting using these drugs (Ferrao et al. 2012; Brooks et al. 2013; Fokas et al. 2014). ATR inhibitors have been shown to have a similar range of activities as the Chk1 inhibitors (Toledo et al. 2011; Huntoon et al. 2013). ATM inhibitors have also been reported to act in combination with chemotherapy and ionizing radiation eliminating cancer cells (Batey et al. 2013; Guo et al. 2014).

3.5.3.3 PARP Inhibitors

Homologous recombination repair utilises both BRCA1 and BRCA2, and *BRCA* mutations cause a high risk of developing breast cancer and susceptibility to ovarian, prostate, pancreatic and male breast cancer (Wooster and Weber 2003). PARP protein is targeted in breast cancer types carrying the mutated form of *BRCA1* and *BRCA2* genes. The loss of BRCA-mediated homologous recombination repair is compensated by BER (Ashworth 2008), and inhibiting BER using PARP inhibitors is selectively lethal to BRCA-mutant cells with only minor effects to wild type cells.

3.5.3.4 Synthetic Lethality

The notion of synthetic lethality was originally used to describe the lethality produced by the simultaneous, but not individual, perturbation of two genes. This concept was then extended to include the disruption of normal protein function of these "partners in crime" through other means such as the action of a chemical compound or environmental changes. Synthetic lethality has served as a tool to get around the issue of undruggable targets in cancer research. Tumour drivers can be challenging to address, especially when the target is a loss of protein function, because activity cannot be rescued through conventional methods such as the use of small molecules or antibodies. An example of therapeutic synthetic lethality based on dysfunctional cell cycle checkpoints is the treatment with histone deacetylase inhibitor (HDACi). HDACi treatment leads to chromatin hyperacetylation which in healthy cells triggers a G2 phase arrest. A high proportion of cancer cell lines do not respond to this DNA insult and progress to mitosis despite the hyperacetylated chromatin state leading to aberrant mitosis followed by multinuclei and micronuclei formation and eventually cell death (Grunstein 1997; Qiu et al. 2000). The apoptosis is a consequence of the ability of HDACi to overcome the spindle assembly checkpoint that should compensate for the failure of G2 phase arrest, with the result of cells undergoing mitotic slippage which triggers apoptosis (Warrener et al. 2003; Stevens et al. 2008; Gabrielli and Brown 2012).

Beyond being exploited as chemosensitizers, defective cell cycle checkpoints are thought to generate alternative potential therapeutic targets. Cell cycle checkpoints are crucial for maintenance of optimal cell survival, therefore when defective, compensatory mechanisms are thought to develop for the cell to cope with abnormal stress. These pathways on which checkpoint defective cells become reliant can be identified using strategies such as high throughput functional genomic screens. This unbiased approach can identify targets that are highly selective and not directly related to the already known player in the synthetic lethal match. For instance, despite the synthetically lethal relationship between BRCA1/2 and PARP1 in *BRCA1/2* mutated tumours being widely studied and successfully applied in cancer therapy, a recent study based on a small interfering siRNA screen identified DDB1 and XAB2 as new synthetic lethal combinations with PARP inhibitors. DDB1 and XAB2 are both involved in DNA damage repair, and these findings could extend the therapeutic use of PARP1 inhibitors to non-BRCA mutant tumours (Lord et al. 2008).

Using similar approaches other latent synthetic lethal relationships have been identified, such as between MSH2 and MLH1, two components of the MMR mechanisms, and DNA polymerases POLB and POLG respectively (Martin et al. 2010), as well as between members of the DNA repair Fanconi anaemia pathway and ATM checkpoint kinase (Kennedy et al. 2007). Somatic Fanconi anaemia mutations have been reported in cancer, and germline mutations to *MSH2*, *MLH1* and to the multiple components of the Fanconi anaemia pathway are thought to predispose to a variety of cancers, supporting the identification of new viable cancer therapeutic targets via unbiased screens.

3.6 Conclusions and Perspectives

Normal cells have evolved a mechanism to protect and maintain the integrity and stability of their genome. This is achieved by the DNA damage response (DDR) pathway, which through cell cycle checkpoint controls temporarily arrests the cell cycle to allow for DNA repair. This mechanism prohibits the amplification of defects leading to abrogated cellular function. Several cancers have been reported to harbor defects in the DDR pathway and checkpoint mechanisms, which explains cellular transformation, genomic instability and increased mutation load of cancer cells. Here we propose to make use of these defective responses by targeting them as a therapeutic approach. The loss of a DNA damage checkpoint in a tumor should provide therapeutic opportunities to inhibit mechanisms that compensate for the defect. Hence, defective cell cycle checkpoints may represent a fertile ground for cancer therapy improvement, either as adjuvants to existing treatments or as a source of potentially targetable dependence mechanisms that are cancer-selective. Synthetic lethality can be exploited for this purpose as a tool for identification of pivotal and highly selective therapeutic candidates.

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Chapter 4
p53 at the Crossroads Between Stress Response Signaling and Tumorigenesis: From Molecular Mechanisms to Therapeutic Opportunities

Luciana E. Giono, M. Fátima Ladelfa, and Martín Monte

Abstract The p53 tumor suppressor is a transcription factor that integrates signals from numerous stress-activated signaling pathways and regulates the expression of specific target genes. p53 activation triggers a variety of cellular responses that ensure tumor suppression, including cell cycle arrest, apoptosis and senescence. In addition, p53 tumor suppressive activity also involves the maintenance of cellular homeostasis through the regulation of metabolic pathways and the protection of stemness.

Mutation of p53 protein or inactivation of the p53 pathway is the most frequent alteration found in human cancer. Loss of p53 function leads to tumorigenesis and is associated with poor prognosis and therapy resistance in cancer patients. Moreover, mutant p53 often exhibits gain of function activities that contribute to the tumoral phenotype.

Over 30 years of basic research on p53 structure and function have placed p53 at the center of cancer investigation. Numerous cellular and mouse models have demonstrated that restoration of p53 function may stop tumor progression or even promote tumor regression. Now, these observations lead to the development of multiple anti-cancer therapeutic strategies that rely on activation of wild-type p53 or reactivation of mutant p53, as well as other p53-based approaches. Rational drug design and functional screenings have allowed for the identification of small molecule compounds, some of which are currently being tested in clinical trials.

L.E. Giono
Laboratorio de Fisiología y Biología Molecular, Departamento de Fisiología y Biología Molecular, IFIBYNE, UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, C1428EHA Ciudad de Buenos Aires, Argentina

M.F. Ladelfa • M. Monte (✉)
Laboratorio de Biología Celular y Molecular, Departamento de Química Biológica, IQUIBICEN, UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, Intendente Güiraldes 2160, C1428EHA Ciudad de Buenos Aires, Argentina
e-mail: mmonte@qb.fcen.uba.ar

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4.1 Introduction

p53 was the first identified tumor suppressor and, if not the most important, it is undoubtedly the most extensively studied. Due to its ability to protect cells from oncogenic progression, p53 has been hailed as the "guardian of the genome", the "cellular gatekeeper", and the "molecular policeman", and it was nominated "Molecule of the year" in 1993 (Lane 1992; Culotta and Koshland 1993). This popularity is certainly not undeserved. In response to numerous types of stress, many of which are associated to the development, progression and dissemination of tumors, p53 triggers a variety of cellular programs that ultimately lead to the suppression of tumor formation. The fact that impairment or loss of p53 function is the most frequent alteration in human cancer underscores this critical role (Levine 1997; Vogelstein et al. 2000; Kandoth et al. 2013).

For many years, p53 was thought to be an only child until two potential family members were identified, p63 and p73. Both proteins exhibit striking sequence similarity and conservation of functional domains with p53. p63 and p73 can bind to DNA through p53 response elements, transactivate some of its target genes, and promote cell cycle arrest and apoptosis following DNA damage. However, neither p63 nor p73 qualify as *bona fide* tumor suppressors: they were not found mutated in human cancer and p63- and p73-knockout mice present severe developmental abnormalities but do not develop spontaneous tumors (Mills et al. 1999; Yang et al. 2000).

From the moment of their identification, the *p63* and *p73* genes were found to contain two separate promoters, internal translation initiation codons and several alternative splice sites at the N- and C-termini, thus encoding multiple isoforms for both proteins (Yang et al. 2002). It took another 5 years before a similar gene structure was recognized for p53, which leads to the expression of at least nine different p53 protein isoforms (Bourdon et al. 2005).

p53, p63 and p73 isoforms have distinct activities. Most notably the N-terminal truncated forms, lacking the transactivation domain, can exert dominant negative effects over the other family members, counteracting their activities (Murray-Zmijewski et al. 2006). Consequently, the interplay between the p53, p63 and p73 proteins and their multiple isoforms has a profound effect on tumorigenesis and therapy outcome (Wei et al. 2012).

4.2 The p53 Tumor Suppressor

4.2.1 p53 Structure

Most of the tumor suppressive activity of p53 has been attributed to its ability to function as a transcription factor, either activating or inhibiting the expression of an

eclectic collection of genes (Riley et al. 2008). The 393-amino acid (aa) protein encoded by the human *p53* gene is organized into several domains that contribute to its transcriptional activity. p53 contains two N-terminal acidic activation domains (aa 1–42 and 43–73), a proline-rich region (aa 61–94) that plays a role in signaling through binding to SH3-containing proteins, and a central DNA binding core (aa 102–292). An oligomerization region is required for tetramer formation (aa 324–355) and subcellular localization is regulated by a cluster of three nuclear localization signals (aa 315–386) and a nuclear export signal (aa 340–351). Finally, a basic regulatory domain located at the C-terminus (aa 363–393) with non-specific DNA binding activity has been shown to negatively regulate binding to DNA by the central core and, therefore, p53 function (Levine 1997). The N-terminal transactivation domain and the C-terminal regulatory domain concentrate most of the numerous sites of post-translational modification that regulate p53 activity (Gu and Zhu 2012) (Fig. 4.1).

Although p53 acts primarily in the nucleus as a transcription factor, transcription-independent activities of p53 in the cytosol promoting apoptosis and inhibiting autophagy have been described (Green and Kroemer 2009).

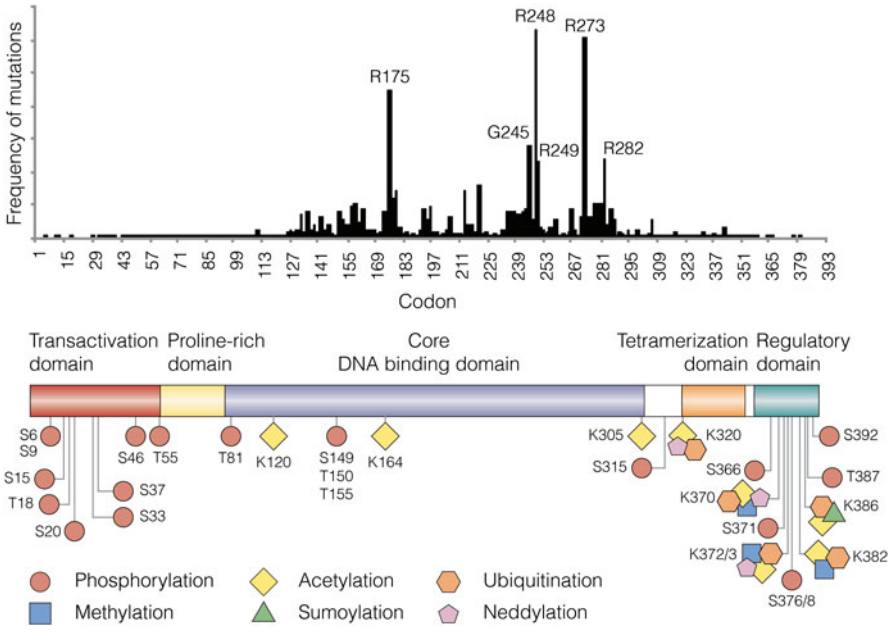

Fig. 4.1 The central part of the figure represents the p53 protein indicating its main domains: the N-terminal transactivation domain, the proline-rich domain, a central DNA binding core and a C-terminal region that includes the tetramerization and the regulatory domains. Multiple residues clustered at the N- and C-termini of p53 that are post-translationally modified by phosphorylation (*circles*), acetylation (*lozenges*), ubiquitination (*hexagons*), methylation (*squares*), sumoylation (*triangle*), and NEDDylation (*pentagons*) are also shown. The histogram of p53 missense mutations (IARC TP53 Database R17, November 2013) highlights that the hotspot mutations correspond to the DNA binding core of the protein (Petitjean et al. 2007)

4.2.2 p53 Regulation

In the absence of stress, p53 is maintained at very low levels due to continuous degradation through the ubiquitin-dependent proteasome pathway. p53 is targeted by numerous E3 ubiquitin ligases, of which Mdm2 plays the most prominent role. Mdm2 keeps p53 activity in check in different ways: Mdm2 associates to the N-terminus of p53, concealing its transactivation domain, and targets C-terminal lysines of p53 for post-translational modification such as ubiquitination and neddylation. Ubiquitination promotes proteasomal degradation and neddylation results in impaired transcriptional activity (Oliner et al. 1993; Moll and Petrenko 2003; Xirodimas et al. 2004) (Fig. 4.2). Mdm2 knockout mice are embryonic lethal due to excessive p53-induced apoptosis and this phenotype is rescued by simultaneous deletion of p53, highlighting the critical role of Mdm2 in the regulation of p53 activity (Moll and Petrenko 2003). p53 is ubiquitinated by nearly a dozen other E3 ligases, including Pirh2, COP1, ARF-BP1 (Lee et al. 2012).

Mdm2 is in turn regulated by p14ARF, a tumor suppressor encoded by an alternative reading frame at the p16INK4A/ARF locus. p14ARF is induced in response to sustained oncogenic signals and protects p53 from Mdm2-dependent degradation by binding to the p53-Mdm2 complex and inhibiting Mdm2 ubiquitin ligase activity (Honda and Yasuda 1999).

MdmX (or Mdm4) is an Mdm2-structural related protein that also binds to the N-terminal domain of p53, blocking its transcriptional activity, and whose deletion produces an embryonic lethal phenotype that is rescued by loss of p53. However, unlike Mdm2, MdmX does not function as an E3 ubiquitin ligase (Marine and Jochemsen 2005).

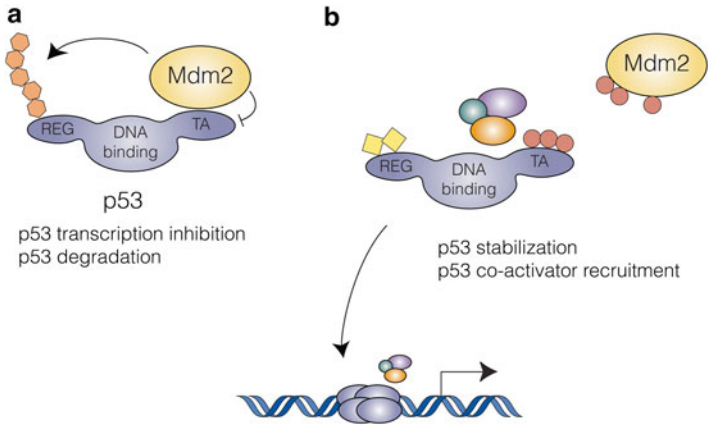

Fig. 4.2 The p53-Mdm2 interaction: (**a**) In unstressed cells, Mdm2 keeps p53 activity in check by two mechanisms: concealment of its N-terminal transactivation domain and targeting to ubiquitin-dependent proteasomal degradation. (**b**) Multiple stress activated signaling pathways result in post-translational modification of both p53 and Mdm2, disrupting their interaction and leading to p53 accumulation and activation

Finally, p53 protein levels are also regulated by ubiquitin-independent proteasomal degradation (Asher et al. 2005) and through translational control (Halaby and Yang 2007).

Upon a variety of stress types, p53 is released from its negative regulators, accumulates in the nucleus and regulates the expression of numerous target genes.

4.2.3 p53 Activation

p53 integrates signals from multiple stress-activated pathways that are triggered in response to oncogene activation, DNA damage, telomere erosion, ribonucleotide depletion, altered mitochondrial and ribosomal biogenesis, nutrient deprivation, hypoxia, and loss of cell contacts (Vousden and Lane 2007) (Fig. 4.3). This integration is mediated by extensive covalent post-translational modification of both p53 and its regulators. p53 contains conserved residues that are modified by phosphorylation, acetylation, methylation, mono- and poly-ubiquitination, sumoylation, neddylation, ADP-ribosylation and glycosylation (Gu and Zhu 2012). The vast majority of these modifications are induced following stress, in a stimuli- and tissue-specific manner; only a few are present in unstressed cells and are removed upon p53 activation.

Numerous studies have reported roles for these modifications in the regulation of almost every aspect of p53 biology: stability, conformation, DNA binding, cellular localization, and protein-protein interaction. However, their absolute requirement

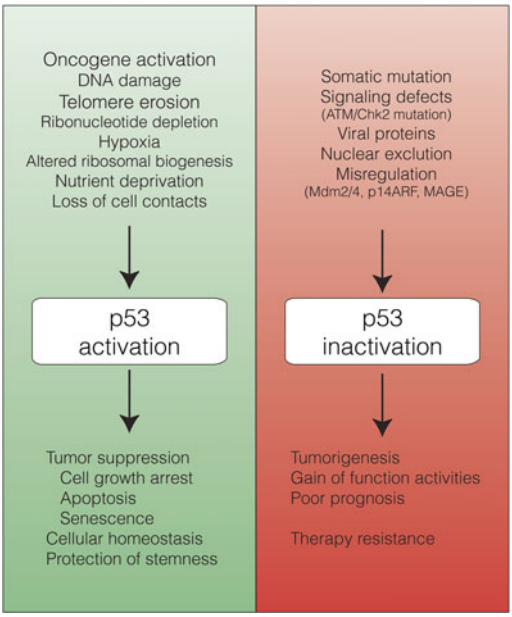

Fig. 4.3 A great variety of cellular stimuli activate p53 that, through the regulation of the expression of multiple target genes, elicits numerous cellular processes responsible of preventing tumorigenesis and ensuring cellular homeostasis. In contrast, inactivation of the p53 pathway or somatic mutation of the p53 protein leads to tumorigenesis and is associated with poor prognosis and therapy resistance. Mut-p53 often exhibits GOF activities that contribute to the tumoral phenotype

for p53 activation has been a source of debate. Mutation studies in mice suggest that there is a significant degree of redundancy among p53 modifications, particularly in the case of phosphorylation. This is reflected in the number of phosphorylation sites, the number of residues phosphorylated by a single kinase, and the number of kinases phosphorylating a single residue. This redundancy might ensure a robust signaling to p53 by stress-activated pathways. Alternatively, it might represent a way to fine-tune the p53 response (Dai and Gu 2010).

Two of the best studied modifications of p53 are phosphorylation at serine 15 and serine 20. Ser15 and Ser20 phosphorylation by DNA damage- and stress-activated kinases (ATM/ATR, Chk1/Chk2, DNA-PK, p38, MAPK, JNK) reduces the interaction of p53 with Mdm2 and enhances the recruitment of transcriptional co-activators (Kruse and Gu 2009). Phosphorylation of Mdm2 by some of the same kinases ensures the disruption of its interaction with p53, possibly as a safety mechanism (Meek and Knippschild 2003) (Fig. 4.2).

p53 is acetylated at multiple C-terminal lysines by p300, CBP (CREB Binding Protein), and other histone acetyltransferases. This modification has been shown to (1) stabilize p53 by competing with ubiquitination of the same C-terminal lysines, (2) enhance sequence-specific DNA binding and transcriptional activity in tissue culture systems, (3) release promoter-bound p53 from a repressed state mediated by Mdm2 and Mdm4 binding and (4) contribute to selectivity in target gene transcription (Kruse and Gu 2009; Dai and Gu 2010). p53 function is therefore regulated by an equilibrium between acetylation and deacetylation, which is maintained by histone deacetylase 1 (HDAC1) and Sirtuin 1 (SIRT1). SIRT1 expression is elevated in many types of cancers and p53 deacetylated at lysine 382 by SIRT1 has a significantly reduced ability to induce apoptosis (Luo et al. 2001).

4.3 Tumor-Suppressive Activity of p53

Appropriate p53 activation is crucial for tumor suppression. This notion is highlighted by different observations, the first of which came from cell biology experiments showing that wild type p53 (wt-p53) can counteract oncogene-induced fibroblast proliferation (Hermeking and Eick 1994). Then, a number of clinical studies evidenced that p53 is mutated in more that 50 % of human tumors (http://www-p53.iarc.fr/) and that patients with Li-Fraumeni Syndrome bearing a mutated-p53 (mut-p53) allele are highly cancer-prone (Malkin 2011). Concluding evidence for a master role of p53 in tumor suppression came from animal models: all mice carrying deleted p53 alleles develop cancer (Purdie et al. 1994; Jacks et al. 1994; Donehower et al. 1992).

The following points of this section underline the molecular mechanisms involving the p53 pathway as a guardian against early events of oncogenesis. Such mechanisms include well-known activities controlling cell proliferation as well as novel mechanisms comprising metabolism regulation and control of stem cells.

4.3.1 A Barrier for Oncogenesis

Oncogene expression boosts cell cycle progression and consequently the activation of p53. This activation depends on the p14ARF/Mdm2 pathway. Oncogene-induced p14ARF expression inhibits Mdm2 E3 ubiquitin-ligase activity and therefore p53 stability (Llanos et al. 2001). In fact, a marked percentage of tumor cells show deficiencies in p14ARF expression or in p53 activity (Honda and Yasuda 1999). If the p53 pathway is intact, oncogene activation triggers the p53 response. Activated p53 will then induce the expression of a variety of target genes involved in cell cycle arrest, senescence or cell death. However, recent data emphasize the relevance of p53 as a regulator of metabolism to exert its full activity as a tumor suppressor (Gu and Zhu 2012).

4.3.1.1 Regulation of Cell Proliferation

A well described p53-dependent way to sustain cell cycle arrest is through the expression of the cdk inhibitors p21 (el-Deiry et al. 1993) and 14-3-3 sigma (Laronga et al. 2000). Oncogene activation can also induce replicative senescence through the p53/PML axis (Pearson et al. 2000). PML itself is a target of p53 (de Stanchina et al. 2004), relocalizes p53 to the PML nuclear bodies causing p53 acetylation and activation, and triggering senescence (Bischof et al. 2002; Pearson et al. 2000).

Depending on cell type and persistence of the stimuli, p53 can efficiently remove potentially dangerous cells by triggering the apoptotic cell death process; an extreme way to eliminate cells with permanent defects considered unfeasible to overcome. In fact, p53 activates a significant set of genes able to induce apoptosis, suggesting that this process is one of the key tools for p53 to eradicate cells with oncogenic potential. Pro-apoptotic genes directly induced by p53 include Bax and Bid, members of the Bcl-2 family (Miyashita and Reed 1995; Sax et al. 2002), PUMA (p53 upregulated modulator of apoptosis) (Nakano and Vousden 2001), NOXA (NADPH oxidase activator) (Oda et al. 2000), and Apaf-1 (apoptotic peptidase activation factor) (Moroni et al. 2001) that activate mitochondrial apoptosis. Also, p53 is able to promote apoptosis by inducing death receptors as Apo1/Fas (Muller et al. 1998), DR4 (Liu et al. 2004) and KILLER/DR5 (Wu et al. 1997).

Therefore, activation of selected p53 target genes involved in the control of cell cycle, senescence or apoptosis is critical to prevent cell transformation (Fig. 4.3). However, more recent findings linking p53 to glucose metabolism, redox balance and autophagy have opened a new window to understand the tumor suppressive activity of p53.

4.3.1.2 Promotion of Normal Cell Metabolism

Glucose Metabolism Metabolism of tumor cells is different from that of normal cells mainly regarding glucose consumption. Tumor cells uptake and metabolize large amounts of glucose to generate lactate in order to obtain energy, even in

normal aerobic conditions (Warburg effect). In this situation, normal cells produce ATP molecules through the more efficient tricarboxylic acid cycle coupled to oxidative phosphorylation. Active glycolysis in tumor cells could allow growth under hypoxia before new blood vessels begin to form in the tumor mass (angiogenesis). However, it seems that tumor cells adopted glycolysis also in normoxic conditions for a fast, instead of efficient, energy production. In fact, activation of oncogenic pathways has been shown to promote the tumor cell metabolic program (glycolysis). The PI3K/AKT pathway is activated by nutrients and promotes cell growth. This pathway is frequently hyperactivated in cancer cells and is also involved in cancer cell metabolism (Vousden and Ryan 2009). In addition, the oncogenic transcription factors c-MYC and HIF regulate genes that accelerate glycolysis (Wise et al. 2008; Yeung et al. 2008), suggesting that metabolic reprogramming is required for cell transformation. Interestingly, regulation of cell metabolism has been included among the p53 tumor-suppressive functions.

p53 can be activated by metabolic stress resulting from low oxygen availability and limited nutrient or energy (Vousden and Ryan 2009). Recent findings have shown that p53 plays a crucial role in controlling glucose metabolism. Activated p53 can regulate the expression of glucose transporters GLUT1, GLUT4 and also GLUT3 (through the NF-kB pathway) (Schwartzenberg-Bar-Yoseph et al. 2004; Kawauchi et al. 2008) and other components of glucose metabolism such as hexokinase II HK2 (Mathupala et al. 2006), TIGAR (TP53-induced glycolysis and apoptosis regulator) (Bensaad et al. 2006) and PGM (phosphoglycerate mutase) (Kondoh et al. 2005). Conversely, p53 induces the SCO2 (cytochrome c oxidase 2) (Matoba et al. 2006) and AIF (apoptosis-inducing factor) genes (Stambolsky et al. 2006) to contribute to oxidative phosphorylation. In this way, p53 promotes normal cell metabolism and ATP production through oxidative phosphorylation and reduces the influx of glucose.

Redox Balance Reactive oxygen species (ROS) are normally produced during general metabolism and, when tightly controlled, they actually promote cell proliferation. However, high levels of ROS cause protein, DNA and lipid oxidation and are associated with aging, cardiopathology and cancer. In order to maintain a correct cellular environment, p53 induces a set of antioxidant proteins (Ladelfa et al. 2012) such as glutathione peroxidase GPX1 (Tan et al. 1999), superoxide dismutase (MnSOD) (Drane et al. 2001), TP53INP1 (TP53-induced protein 1) (Cano et al. 2009) and members of the sestrin family, Sesn1 and Sesn2 (Budanov et al. 2004). In contrast, induction of p53-dependent apoptosis has a strong oxidative component since most of the p53 targets that induce apoptosis through the intrinsic mitochondrial pathway produce high levels of ROS (Ladelfa et al. 2012). In summary, p53 appears to play an important role in promoting normal cell metabolism by regulating glycolysis and the oxidative balance. Importantly, a mut-p53 unable to induce apoptosis due to lack of key lysines, maintained its ability to regulate metabolism and displayed tumor suppressive function (Gu and Zhu 2012) stressing the relevant role of p53 in controlling metabolism.

Autophagy Another metabolic process in which p53 is involved is autophagy (macro-autophagy), a way to obtain energy through controlled degradation of organelles and proteins into lysosomes when nutrients are scarce. Therefore, through the autophagic process, cells replenish energy reserves and promote cell survival. p53 plays a dual role in this process, by inducing autophagy from the nucleus and repressing it in the cytoplasm (Comel et al. 2014). The main pathway controlled by p53 is the AMPK-mTOR axis. This pathway inhibits autophagy and, in turn, p53 inhibits the pathway at multiple levels. p53 was shown to activate transcription of several genes whose protein products can directly or indirectly regulate signaling from AMPK to mTOR: TSC2, beta-1 and beta-2 subunits of AMPK and sestrins 1 and 2 (Feng et al. 2005, 2007; Budanov and Karin 2008). In addition, DRAM (damage-regulated autophagy regulator), another p53 target gene, is a lysosomal protein that activates autophagy, linking this process to apoptosis (Crighton et al. 2006). In unstressed cells, p53 has a cytoplasmic component that counteracts autophagy. Forced depletion of p53 or cell starvation, which physiologically induces p53 degradation, promotes autophagy. This mechanism was observed only in cells bearing wt-p53 and is associated to downregulation of the key autophagy protein LC3. By playing this role p53 could balance the energy requirements both when the autophagy rate is low or high (Maddocks and Vousden 2011; Scherz-Shouval et al. 2010).

All the above mentioned data point to p53 as pivotal protein able to sense a wide range of stimuli to trigger responses focused on preserving the proper cell function. Once this balance is broken, the p53 response becomes focused on the elimination of potentially dangerous cells.

4.3.2 p53 Controlling Stemness

In addition to the aforementioned role in differentiated cells, p53 is also involved in the protection of stem cells (SCs). SCs are undifferentiated cells capable of self-renewal and generating specific cell lineages through asymmetrical cell division. Embryonic stem cells (ESCs) can differentiate into ectoderm, mesoderm and endoderm lineages, while adult stem cells (ASCs), which are found in many tissues, regenerate the corresponding tissue-specific cells. This hierarchical and one-way order puts SC at the top, followed by somatic cells with diverse degree of differentiation. However, breakthroughs in the SC field showed the possibility of inducing pluripotent SC (iPSCs) from differentiated mouse embryonic and adult fibroblasts, through the ectopic expression of four proteins: Oct3/4, Sox2, KLF4 and c-Myc (Takahashi and Yamanaka 2006).

Earlier studies showed that the classical p53 response is reduced in mouse ESCs (Rogel et al. 1985; Sabapathy et al. 1997; Han et al. 2008; Aladjem et al. 1998). This behaviour presents a paradox, given the high cell division rate of SCs. Many recent studies shed light on this field indicating that p53 response in SCs is responsible for suppression of self-renewal and induction of differentiation when genomic stability

is compromised after DNA damage (Aloni-Grinstein et al. 2014). Activated p53 in SCs binds to the promoters and represses the expression of Nanog and Oct4, two well-known markers of SCs and key proteins for the maintenance of the self-renewal and undifferentiated state. Thus, upon DNA damage, repression of these two genes by p53 forces SCs differentiation (Lin et al. 2005). In-deep studies of p53 in SCs identified a number of target genes involved in development and differentiation such as those belonging to the FOX, SOX, TBX, CBX and homeodomain families (Akdemir et al. 2014; Morey and Helin 2010).

Mesenchymal SCs (MSCs) are a type of ASC that generate different mesodermal cells when they differentiate. p53 point mutations were found in aged MSCs along with embryonic markers, suggesting that non functional p53 could be related to tumor development of mesenchymal origin (i.e., sarcoma) during ageing (Li et al. 2007). Transformation or dedifferentiation of ASCs can lead to the formation of cancer stem cells (CSCs). These cells are reservoirs of cancer cells with similar features (self-renewal and generation of more differentiated tumor cells) to that of normal SCs (Aloni-Grinstein et al. 2014). Using mouse models of MSCs, it was demonstrated that alterations in the p53 pathway but not the retinoblastoma (pRb) pathway is associated with sarcomatogenesis (Rodriguez et al. 2012; Rubio et al. 2010) and that fibrosarcomas derived from aged animals could be originated from a MSCs bearing mut-p53 (Li et al. 2007).

4.4 Deficient p53 Activity in Tumor Cells

It is now clear that defects in any step of the p53 pathway can facilitate tumor promotion. In addition to being a key protein in the control of carcinogenesis, proper p53 activation is also an important aspect of cancer therapy. Radiotherapy and chemotherapy rely mainly on the induction of (selected) DNA damage-induced apoptosis or at least on inhibiting cell proliferation. Therefore, induction of p53 by ionizing radiation (radiotherapy) or DNA-damaging agents (chemotherapy) mostly correlates with good therapy outcome.

In ovarian cancer patients, p53 alterations correlate with resistance to platinum-based (cisplatin or carboplatin) chemotherapy, early relapse and shortened overall survival (Reles et al. 2001; Shelling 1997). Non response to purine analogs therapy (fludarabine or pentostatin) and poor survival were reported in chronic B-cell leukemia patients with p53 gene deletion (Dohner et al. 1995). p53 mutations are also associated with poor response to chemotherapy and radiotherapy in colorectal and gastric cancer patients (Hamada et al. 1996). For other cancers, resistance to therapy due to non functional p53 activity is not well established and seems to depend on both cellular context and chemotherapeutic drugs.

Inadequate p53 function in tumor cells is mainly caused by gene mutation, but it has been also shown that oncogenic proteins can target p53 activity (Fig. 4.3). That knowledge obtained from basic cancer research was essential to develop p53-based therapies. Both scenarios exhibit a degree of complexity and are described here.

4.4.1 Mutant p53 in Tumors

A small number of tumors have no detectable p53 protein expression due to frameshift or nonsense mutations. However, approximately 75 % of tumor-associated alterations in p53 are missense mutations, with a single amino acid change in the p53 protein. These mutants are expressed at very high levels in cancer, suggesting that their expression can confer advantages to tumor cells (Petitjean et al. 2007). Indeed, 20 years ago, Dittmer and co-workers reported that the introduction of mut-p53 into p53 null cells results in a new phenotype and suggested that mut-p53 can gain a novel transforming function (Dittmer et al. 1993). Since then, several cell culture assays have demonstrated that different mut-p53 proteins acquire gain of function (GOF) activities such as promotion of cell survival, drug resistance, anchorage-independent growth, increased colony formation and genomic instability among others (Muller and Vousden 2014) (Fig. 4.3).

Consistent with p53 functioning primarily as a transcription factor, the vast majority of the missense mutations observed in p53 lie in the core DNA binding domain. Mutations frequently found in p53 (hotspot mutations) can be classified as class I/"DNA contact" mutations (R248, R273) or class II/"conformational" mutations (R175, R245, R249, R282) that disrupt or destabilize p53 structure (Brosh and Rotter 2009; Petitjean et al. 2007; Muller and Vousden 2013) (Fig. 4.1).

Mice models were developed in order to study mut-p53 GOF in a physiological context, allowing more properly assessment of cancer parameters such as metastatic potential, spectrum of tumor generation, angiogenesis and tumor latency. Knock-in mice in which the endogenous p53 mouse allele was replaced with orthologs of specific human hotspot p53 mutants, such as $p53^{R172H/-}$ (corresponding to human $p53^{R175H}$) and $p53^{R270H/-}$ (corresponding to human $p53^{R273H}$), developed a broadened spectrum of tumor and showed increased metastatic potential compared to $p53^{-/-}$ mice (Lang et al. 2004), supporting the notion of a GOF mechanism for these mutants.

Similar studies were performed in a *hu*manized *p*53 *k*nock-*i*n (HUPKI) mouse model (Luo et al. 2001). Of special interest, $p53hupki^{R248Q/-}$ mice showed the most potent mut-p53 GOF reported to date, displaying diminished survival and decreased tumor latency compared to $p53^{-/-}$ mice (Hanel et al. 2013).

Lessons learnt from cell culture assays and knock-in mouse models support the notion that different mut-p53 promote different oncogenic responses, not only by losing wt-p53 function but also by gaining novel pro-oncogenic functions.

4.4.2 Inactivation of Wild Type p53

In addition to mutations, p53 can also be inactivated by oncogenic viral proteins encoded by small DNA viruses. In fact, p53 was discovered as a 53 kDa protein complexed to the SV40 large T viral oncoprotein (LT) (Lane and Crawford 1979).

LT directly binds the p53 DNA binding domain, thus blocking p53 interaction with DNA (Ali and De Caprio 2001). High risk human Papilloma Virus E6 oncoprotein interacts with E6AP (E6 associated protein) and p53 to promote ubiquitin-mediated degradation of p53 (Huibregtse et al. 1993; Scheffner et al. 1990). In adenovirus transformed cells, p53 inhibition of cell proliferation and induction of apoptosis are blocked by the E1B-55K protein. This protein binds with high affinity to the p53 transcriptional activation domain, probably sterically blocking interactions of p53 with its coactivators (Berk 2005).

Currently, growing evidence suggest that, similar to mut-p53 proteins, viral oncoproteins-p53 complexes could contribute to tumorigenesis through GOF mechanisms. According to this, new transcriptional and biological activities, such as the activation of the IGF-I signalling pathway by the LT-p53 complex, have been reported (Bocchetta et al. 2008).

Another line of attack to wt-p53 comes from cellular proteins highly expressed in tumor cells. For example, high levels of Mdm2 are commonly observed in human cancers due to gene amplification, elevated transcription, increased mRNA stability, enhanced translation, and altered post-translational modifications (Riley and Lozano 2012). High levels of Mdm4 protein are also found in a variety of human cancers mostly due to Mdm4 gene amplification (Markey 2008). Conversely, loss of p14ARF, which antagonizes Mdm2, allows tumor development without loss of wt-p53 (Stott et al. 1998).

Analysis of tumors and tumor cell lines that retain wt-p53 pointed to nuclear exclusion as yet another mechanism of tampering with p53 function, particularly in breast carcinomas and neuroblastomas (Moll et al. 1992, 1995). Although Mdm2 amplification was not observed in those samples, subsequent studies showed that Mdm2 might play a significant role in the cytoplasmic p53 phenotype (Lu et al. 2000).

Finally, the tumor-specific MAGE-I (Melanoma antigen gene) proteins, especially Mage-A proteins, negatively regulate p53 activity possibly by both direct binding to p53 (Marcar et al. 2010) or by recruiting HDACs (Monte et al. 2006) or KAP1 (Doyle et al. 2010; Yang et al. 2007) proteins to p53-containing complexes (Ladelfa et al. 2011). As MAGE-I proteins are expressed in humans cancers of different lineages, interfering with its expression or function could have an impact on the treatment of a wide range of human cancers, especially those harbouring wt-p53.

4.5 p53, a Key Target for Cancer Therapy

Many aspects of p53 biology put it at the center of cancer therapy treatments. As mentioned earlier, loss of p53 function is the most frequent alteration in human cancer, highlighting the unquestionable role that p53 plays in tumorigenesis. At the same time, loss of p53 also contributes to therapy resistance. Drug dose escalation is one strategy to improve the therapeutic outcome in p53-negative tumors but this also enhances the toxicity in normal tissues, in particular in those that are

highly sensitive to p53 induced apoptosis, generating chemotherapy side effects. In summary, p53 is both at the origin of the disease and a challenge to its treatment. In view of all this, p53 represents an extremely attractive and highly strategic target for cancer treatment.

4.5.1 Proof-of-Principle and Strategy to Restore p53 Function

A key question arises before pursuing any p53-based cancer therapy. Once mutation or inactivation of p53 has led to tumor formation, will restoring p53 function be sufficient to stop its progression or even promote tumor regression?

Elegant mouse models were engineered to answer this question that provided promising proof-of-principle results. Through different mechanisms, mainly apoptosis or senescence, re-expression of endogenous p53 was able to cause stasis or regression of the established tumors (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007). These observations bolstered research on multiple approaches to reinstate p53 function in tumor cells and hundreds of clinical trials looking into or targeting p53 are currently ongoing (http://clinicaltrials.gov) (Kenzelmann Broz and Attardi 2010; Hoe et al. 2014).

p53 deficiency in human cancer can be classified into three categories: loss of p53, mutation of p53 and inactivation of wt-p53. As previously discussed, p53-null tumors should be considered separately as missense mutations of p53 can have a substantially different effect on cellular responses compared to deletion or nonsense mutations. Anti-cancer therapeutic strategies based on targeting p53 can similarly be divided into corresponding categories: activation/re-introduction of wt-p53 and reactivation of mut-p53 (Frezza and Martins 2012).

4.5.1.1 Activating Wild Type p53

Current p53 gene therapy relies mostly on p53 delivery by replicative-deficient adenoviruses (Ad-p53), which had shown dramatic apoptotic responses and tumor regression in cell culture and rodent models. Since 2003, Ad-p53 Gendicine (Shenzhen SiBiono GeneTech, China) is being applied in China in combination with radiation therapy for the treatment of head and neck cancer and hepatocellular carcinoma. Ad-p53 Advexin (INGN-201), developed by Introgen Therapeutics, is yet to be approved by the U.S. Food and Drug Administration and several clinical trials are underway to test the efficacy of this and other Ad-p53 (SCH-58500), alone, in combination therapy, or following surgery (Kim and Dass 2011).

Attenuation of the p53 response in tumors expressing wt-p53 can result from alterations in p53 regulatory proteins. Basic research conducted over the past 20 years has singled out Mdm2, as well as Mdm4, as the major negative regulators of p53 with oncogenic potential. The efficient and natural strategy to activate p53 shared by p14ARF, some ribosomal proteins or the DNA damage-induced

post-translational modifications, consists in disrupting the Mdm2-p53 interaction. Based on this, the use of small molecule compounds, antisense therapy and stapled peptides committed to interfere with Mdm2-p53 complex is a key strategy currently explored in order to activate p53 (Wade et al. 2013).

X-ray crystallography of the p53-Mdm2 interface combined with site-directed mutagenesis experiments and studies using peptidic inhibitors showed that their interaction was a suitable drug target and allowed to derive pharmacophore models for this protein-protein interaction (Chene 2004; Shangary and Wang 2009). Together with high throughput screenings, these models were used in structure-based de novo design and computational 3D screenings of the National Cancer Institute database to identify hundreds of potential Mdm2 inhibitors (Essmann and Schulze-Osthoff 2012) (Table 4.1). RG7112 (Hoffman-La Roche), a Nutlin 3a potent derivative, and the spiro-oxindole MI-219 are some of these small molecule compounds that have progressed into advanced preclinical development or early phase clinical trials.

One of the obstacles for full p53 activation in response to these compounds is the negative regulation by MdmX. Selective small molecule inhibitors of the MdmX-p53 interaction, small molecule and staple peptide dual Mdm2/MdmX-p53 antagonists, Mdm2 ubiquitin ligase inhibitors and compounds that inhibit the stability of the MDM proteins are under study (Wade et al. 2013).

Finally, other approaches to enhance wt-p53 activity include: inhibiting the p53 deacetylases SIRT1 and SIRT2; blocking nuclear export, thus protecting p53 from Mdm2-mediated degradation; activating p53 through non-genotoxic drugs that cause nucleolar disruption such as low doses of Actinomycin D, among others (Lane et al. 2010).

4.5.1.2 Reactivating Mutant p53

Reactivation of mut-p53 is based on the selection of small molecules able to change p53 function from mutant to wild type.

PRIMA-1 (p53 reactivation and induction of massive apoptosis) was identified in a functional screening and shown to restore DNA binding to several p53 mutant forms (Selivanova 2010). PRIMA-1 and its optimized version PRIMA-1^{Met} displayed potent anti-tumorigenic activity in animal models, alone or in combination with chemotherapy. A structural analog, APR-246, has entered in phase I/II clinical trials. An in silico screening using the crystal structures of several p53 mutant proteins led to the identification of PhiKan083, a small molecule compound that can selectively reactivate the Y220C p53 mutant (Boeckler et al. 2008).

Mut-p53 proteins are often abundant with the additional burden of the many GOF properties that they may display, including detrimental dominant-negative interactions with the p53 siblings p63 and p73, as well as re-targeting of mut-p53 to promoters of other genes (Freed-Pastor and Prives 2012; Muller and Vousden 2014).

RETRA (reactivation of transcriptional reporter activity) was shown to induce p53-like activity in cells and tumor regression in mouse xenograft models. Rather

Table 4.1 p53 targeting and p53-based therapies

Mechanism of action	Compound	Type	Stage
p53 Gene therapy	Advexin (INGN-201)	Adenovirus	Phase I/III
	Gendicine (China)		Phase IV (China)
	SCH-58500		Phase I/III
Mdm2/MdmX antagonists	RG7112 (nutlin derivative)	Small molecule	Phase I
Mdm2-p53 interaction	MI-219/-319	Small molecule compounds	Preclinical
	RITA, AM-8553, terphenyl 14		
	Sulfonamide I (NSC 279287)		
	Quinolinole (NSC 66811)		
	Benzodiazepinedione (TDP521252, TDP665759)		
	PXn727/822, isoindolinone 74a		
Mdm2 E3 ligase activity	HLI98, MPD, MEL23, MEL24	Small molecule	
Binding of Mdm2-p53 to proteasome	JNJ-26854165	Small molecule	Phase I
Mdm2 antisense	GEM240	Oligonucleotide	Preclinical
MdmX-p53 interaction	WK298	Small molecule	Preclinical
	SJ-172550		
MdmX destabilization	NSC207895	Small molecule	Preclinical
	17-AAG (HSP90 inhibitor)		
Dual Mdm2/MdmX antagonists	RO-5963	Small molecule	Preclinical
	SAH-p53-8	Peptidic	Preclinical
	PMI peptide, pDI peptide		
Nuclear export inhibitors	Leptomycin B	Small molecule	Preclinical
Sirtuin inhibitors	Tenovin-1/-6		
	Actinomycin D		
Reactivation of mut-p53	APR-246 (PRIMA-1 MET)	Small molecule	Phase I/II
	MIRA-1/MIRA-3	Small molecule	Preclinical
	STIMA-1, ellipticine, CDB3		
	SCH529074, PhiKan083		
	CP-31398, NSC319726		
mut-p53-p73 interaction	RETRA, SIMP	Small molecule	Preclinical
Oncolytic adenovirus	ONYX-015/oncorine (H101) (China)	Adenovirus	Phase I/II
	ColoAd1		
	p53-SLP	Peptide	Phase I/II
p53 vaccination	INGN-225	Cellular	Phase II
	ALT-801	Fusion protein	Phase I/II
Cyclotherapy	Nutlin + BI-2536 (Plk1 inhibitor)		Phase I
	Nutlin + VX-680 (Aurora kinase inhibitor)		

than reactivating mut-p53, RETRA was found to release p53 from inactive mut-p53-p73 complexes.

Alternative approaches are being explored to deal with mut-p53 that try to eliminate these proteins by promoting their degradation (Muller and Vousden 2014).

4.5.2 Other p53-Based Strategies

As no rock is left unturned in this field, a few developments do not attempt to restore p53 function in tumor cells but still exploit p53 for therapeutic gain. Conditionally replicating adenoviruses have been engineered to only replicate in and then lyse p53-deficient tumor cells, while leaving normal cells unharmed. Oncorine (ONYX-015/H101) was approved by Chinese SFDA and several phase I/II clinical studies using ColoAd1 (PsiOxus Therapeutics) are currently underway.

A radically different approach uses vaccination with p53-derived peptides to target tumor cells. Mutation of p53 might alter its antigenicity and, more importantly, is often associated with high protein levels. p53 becomes then immunogenic and anti-p53 antibodies have been detected in patients with different types of cancer. Synthetic p53-derived peptides (p53-SLP, Isa Pharmaceuticals), dendritic cell-based p53 vaccines (INGN-225, Introgen) and an IL-2/T-cell receptor fusion protein targeting p53 (ALT-801) are being trialed in combination with adjuvant and/or chemotherapy.

Finally, cyclotherapy is an emerging strategy to selectively kill proliferating tumor cells while protecting normal cells from the side effects of cytotoxic therapy. The concept behind is that low doses of non-genotoxic p53 activators will induce a transient cell cycle arrest in normal cells, whereas mut-p53 cancer cells will continue to proliferate. Drug withdrawal following treatment with chemotherapeutic agents (S- and M-phase poisons) should allow normal cells to re-enter the cell cycle spared from cytotoxic effects (Rao et al. 2013). Combination of nutlin with a variety of mitotic poisons, DNA damaging agents, and kinase inhibitors among others, have been experimentally studied and await further testing in *in vivo* models (van Leeuwen et al. 2012; Hoe et al. 2014).

4.5.3 Future Directions and Expectations

Over 30 years of basic cancer research have paved the way for the development of a panoply of candidate-driven strategies, structure-based rationally designed drugs and combinatorial approaches that exploit and translate the immense accumulated knowledge on the life and work of p53 into cancer therapies (Stegh 2012) (Fig. 4.4).

It seems important to mention that many of the small molecule compounds designed as anti-cancer therapies proved to be, in turn, invaluable tools to answer key basic science questions regarding p53 function and regulation which may result

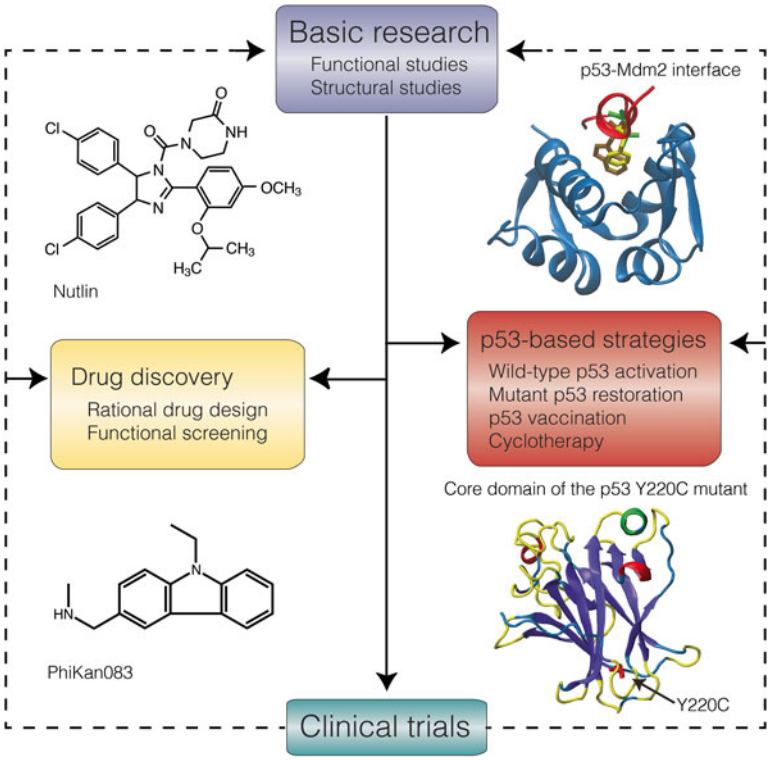

Fig. 4.4 Over 30 years of p53 basic research have led to the development of numerous anti-cancer therapeutic strategies which, in turn, represent exceptional tools to address key questions regarding p53 biology. The insets illustrate the translational process for two strategies of p53-based approaches: (*top*) activation of wt-p53: structure of the p53-Mdm2 interface which is disrupted by the small molecule Nutlin, and (*bottom*) reactivation of mut-p53: structures of the Y220C p53 mutant and PhiKan083, a carbazole derivative that stabilizes it (Boeckler et al. 2008)

in the development of more and novel strategies, thus feeding the cycle: from the bench to the clinic and back again.

A lesson to draw from research on p53 is the importance of context. p53 is at the center of a highly convoluted and delicately balanced signalling network, where novel roles of p53 are almost periodically uncovered. p53 activity is modulated by countless post-translational modifications, a multitude of positive and negative regulators and influenced by the interactions with its multiple isoforms and family members (Machado-Silva et al. 2010). The p53 response is stimuli-dependent and tissue-specific, as is the expression levels of many of these activators and inhibitors. The nature of the p53 mutation as well as single nucleotide polymorphisms in the p53 pathway can impact on the response to the different compounds used or studied for cancer treatment (Grochola et al. 2010). It is therefore critical to take all these variables into account when designing clinical trials and developing improved p53-based cancer therapies.

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Chapter 5
Sirtuins as a Double-Edged Sword in Cancer: From Molecular Mechanisms to Therapeutic Opportunities

Núria Sima, Laia Bosch-Presegué, and Alejandro Vaquero

Abstract Sirtuins are a family of NAD^+-dependent enzymes that participate in cellular adaptation to stress, particularly to stress associated with changes in energy metabolism. Sirtuins originated in prokaryotes, participating in vitamin-B12 metabolism, and later appeared in early eukaryotes with an additional function: protecting the genome from endogenous or exogenous stress. This early origin places Sirtuins at the intersection of various stress-related pathways within the complex signaling network that regulates stress response. Thus, Sirtuins are critical in many human pathologies—especially cancer and aging. In cancer, Sirtuins can act as tumor promoters or suppressors, depending on the cell type and the functional context. Therefore, they are promising targets for cancer therapeutics and, according to some evidence, might serve as biomarkers of tumor status and stage.

Keywords SIRT1-7 • Genotoxic stress • NAD • Metabolic stress • Oxidative stress • Posttranslational modification • Facultative heterochromatin • Constitutive heterochromatin • Histone deacetylase • ROS • Tumor suppressor • Oncogene • Genome stability • Chromatin • DNA damage repair • Cell-cycle • Apoptosis

5.1 Introduction

Among the factors that regulate stress response in eukaryotes, the members of the Sir2 family (or *Sirtuins*) appear to be unique: they not only regulate this response at different levels, but are also effectors of it. The functional versatility of these deacetylases and ADP-ribosyltransferases greatly derives from the fact that they require NAD^+ as enzymatic cofactor. Accordingly, researchers have suggested that Sirtuins

N. Sima • L. Bosch-Presegué • A. Vaquero (✉)
Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Av. Gran Via de l'Hospitalet 199-20308907 L'Hospitalet de Llobregat, Barcelona, Spain
e-mail: avaquero@idibell.cat

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might be sensors of metabolic and energetic imbalance, although this has not been demonstrated (Imai et al. 2000; Vaquero 2009).

Sirtuins first arose in prokaryotes, participating in vitamin-B12 metabolism, and later appeared in early eukaryotes, showing an additional function: participating in the crosstalk between environment and genome. Given their early origin, Sirtuins are at the crossroads of various stress pathways: they participate in metabolic homeostasis, gene expression, chromatin structure dynamics, DNA repair, cell-cycle control, inflammation and apoptosis, among other areas. Throughout evolution, Sirtuins have diversified in number, from a single prokaryotic member to seven mammalian members, *SIRT1-7* (Frye 2000). Likewise, their scope of functions, substrates and cellular localizations (from the nucleus to mitochondria) has expanded (Table 5.1).

Sirtuins seem to be focused on three related forms of stress, all closely related to carcinogenesis (Yang et al. 2003): *metabolic* (excluding oxidative), *oxidative* and *genotoxic* (Bosch-Presegué and Vaquero 2013) stress. In metabolic stress, Sirtuins promote metabolic homeostasis at the cellular and systemic levels and are intimately linked to the endocrine system—particularly, to the insulin/insulin-like growth factor associated pathways. An interesting example of this type of stress is *calorie restriction* (*CR*), a 30–50 % reduction in food intake that has been shown to extend lifespan in many organisms and to improve human pathologies such as cancer or diabetes (Qiu et al. 2010). Some studies report that Sirtuins partially mediate the beneficial effects of CR on aging and age-related diseases in mammals.

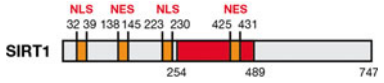
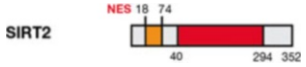
Oxidative stress refers to the conditions generated by reactive oxygen species (ROS) produced by the mitochondrial respiratory chain, peroxisomes, lipooxygenases, radiation, chemotherapeutics or environmental toxins. Interestingly, CR indisputably reduces production of ROS (Vaquero and Reinberg 2009). Paradoxically, although CR and ROS are opposing phenomena, they both activate Sirtuins.

Finally, *genotoxic stress* encompasses the genomic damage caused by a harmful endogenous or exogenous agent, including metabolic products, ROS, radiation (e.g. UV or ionizing), chemotherapeutics or environmental toxins. These agents damage DNA in different ways, including single-strand breaks (SSBs) and double-strand breaks (DSBs), and drastically alter chromatin organization and structure as well as the cell cycle (Yang et al. 2003). In genotoxic stress response, Sirtuins regulate chromatin structure maintenance, DNA repair, gene expression, cell-cycle progression and apoptosis.

5.2 Activation of Sirtuins by Stress

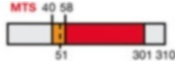
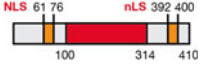
Given their critical role in stress response, Sirtuins are activated and regulated by myriad signaling pathways. However, current knowledge on these mechanisms is chiefly restricted to SIRT1 and SIRT6. The mechanisms identified to date include gene expression, RNA stability and post-translational modifications (PTM).

Table 5.1 Sirtuin localization, function and targets

Conserved catalytic domain	Activity	Localization	Principal targets under stress	Regulation by CR/ROS	Sirtuin function	References
	Deacetylase	Nucleus/ cytoplasm	H4K9Ac, H4K16Ac, SUV39h1, XPA, XPC, NBS1, hMOF, TIP60, WRN, Ku70, Rb, FOXO, HIF-1α, p53	Up-regulated in WAT and muscle, down-regulated in liver and pancreas/ up-regulated	Heterochromatin structure maintenance, ribosome biosynthesis, DNA repair, cell cycle control, apoptosis	Bosch-Preseguè et al. (2011), Jeong et al. (2007), Jin et al. (2007), Murayama et al. (2008), Peng et al. (2012), Vaquero et al. (2007), Vaquero et al. (2004), Wong and Weber (2007), and Yuan et al. (2007)
	Deacetylase	Cytoplasm/ nucleus	H4K16Ac, H3K56Ac, PR-SET7, CDH1, CDC20, CDK9, FOXO, p53	Up-regulated/ up-regulated	Cell cycle control, apoptosis	Dryden et al. (2003), Kim et al. (2011), North and Verdin (2007), Serrano et al. (2013), and Vaquero et al. (2006)
	Deacetylase	Mitochondrial matrix/nucleus	AceCS2, IDH2, MnSOD, p53, Ku70	Up-regulated/ Up-regulated	Metabolism homeostasis, ROS levels control, apoptosis	Haigis et al. (2012) and Onyango et al. (2002)

(continued)

Table 5.1 (continued)

Conserved catalytic domain	Activity	Localization	Principal targets under stress	Regulation by CR/ROS	Sirtuin function	References
SIRT4 	ADP-ribosyltransferase	Mitochondrial matrix	GDH, MCD	Down-regulated/ND	Metabolism homeostasis	Ahuja et al. (2007), Jeong et al. (2013), Jeong et al. (2014), and Laurent et al. (2013)
SIRT5 	Deacetylase/ deacylase	Mitochondrial matrix	CPS1	Up-regulated/ ND	Metabolism homoeostasis, ROS detoxification	Du et al. (2011), Matsushita et al. (2011), and Peng et al. (2011)
SIRT6 	ADP-ribosyltransferase/ deacetylase/ deacylase	Nucleus	H3K9Ac, H3K56Ac, PARP1, CtiP, SNF2H, NF-kB, p53, p73	Up-regulated/ ND	Telomere maintenance, DNA repair, metabolism homeostasis ribosome biosynthesis, apoptosis	Jiang et al. (2013), Kaidi et al. (2010), Mao et al. (2012), Michishita et al. (2008), Mostoslavsky et al. (2006), and Tennen et al. (2010)
SIRT7 	Deacetylase	Nucleolus	H3K18Ac, RNA Pol, p53	Up-regulated/ ND	rDNA expression, ribosome biogenesis, apoptosis	Barber et al. (2012), Ford et al. (2006), and Kiran et al. (2013)

CR calorie restriction, *MTS* mitochondrial target sequence, *ND* not determined, *NES* nuclear exportation sequence, *NLS* nuclear localization sequence, *nLS* nucleolar localization sequence, *ROS* reactive oxygen species

5.2.1 *Modulation of Sirtuin Gene Expression and RNA Stability*

Expression of all seven eukaryotic Sirtuins is tightly regulated by different combinations of transcription factors. For instance, CR induces expression of SIRT1-3 and SIRT6, inhibits expression of SIRT4, and does not affect expression of SIRT5 (Qiu et al. 2010). CR activates the expression of SIRT1 in white adipose tissue (WAT) and muscle accordingly with the higher levels of NAD^+ in the cytoplasm and nucleus. In contrast, it represses the expression of SIRT1 in the liver and pancreas, where, compared to the aforementioned tissue types, the NAD^+/NADH ratio is lower in the nucleus and cytoplasm, and higher in the mitochondria.

Expression of SIRT1, the best-studied Sirtuin, is regulated by various transcription factors involved in cell growth, differentiation and migration, in stress resistance and in metabolism. Interestingly, most of these different forms of SIRT1 regulation exhibit a feedback loop between a given factor and SIRT1. These factors include the tumor suppressor p53, which is involved in cell-cycle checkpoint regulation, stress response and apoptosis. Two functional p53 binding sites have been identified in the SirT1 promoter. In response to cellular access to nutrients, p53 regulates SIRT1 expression in different directions. In nutrient-deprived mammalian cells, FOXO3 transcription factor forms a complex with p53, which is recruited to the Sirt1 promoter sites, stimulating SIRT1 expression (Nemoto et al. 2004). Another interesting factor is the redox sensor Carboxy-terminal of E1A-binding protein (CtBP), which can directly sense changes in the NAD^+/NADH ratio and might be among the earliest acting sensors of the response. In response to metabolic stress, Sirt1 gene expression is regulated by CtBP and the tumor suppressor Hypermethylated in cancer-1 (HIC1). Cellular redox changes sensed by CtBP alter its affinity for HIC1, thereby diminishing CtBP recruitment to the Sirt1 promoter and derepressing Sirt1 expression (Zhang et al. 2007). In turn, SIRT1 interacts with and deacetylates HIC1, enabling its sumoylation and increasing its transcriptional repression activity (Stankovic-Valentin et al. 2007). In contrast, under normal nutrient conditions, p53 mediates Sirt1 gene repression in cooperation with HIC1 (Chen et al. 2005).

Another important regulator of SIRT1 gene expression is E2F1, an oxidative stress and DNA damage-response transcription factor that controls G1/S phase progression. E2F1 binds directly to the SIRT1 promoter and, in cells treated with the topoisomerase II inhibitor etoposide, upregulates expression of SIRT1. Phosphorylation of E2F1 by the stress-response kinase Ataxia telangiectasia mutated (ATM) seems to be necessary for E2F1-mediated regulation of Sirt1 gene expression. In turn, SIRT1 interacts with and deacetylates E2F1, inhibiting its pro-apoptotic activity and inducing cell proliferation (Wang et al. 2006). Another feedback of SIRT1 has been reported with the oncogene c-Myc, which upregulates Sirt1 expression. In turn, SIRT1 deacetylates c-Myc, leading to its degradation and inhibiting cellular proliferation (Yuan et al. 2009).

Interestingly, E2F1 and p53 also regulate SIRT1 at the translational level. The microRNAs miR-34a and miR449a, which are targets of p53 and E2F1, respectively, both inhibit SIRT1 expression (Yamakuchi et al. 2008). Moreover, SIRT1 cellular levels are also regulated by the RNA binding protein HuR, which associates with SIRT1 mRNA, leading to increased Sirt1 mRNA stability and consequently, to increased levels of SIRT1 protein (Abdelmohsen et al. 2007).

SIRT6 is a histone deacetylase and mono[ADP-ribosyl]transferase involved in different mechanisms of genome protection during stress. Under nutritional stress, SIRT1 forms a complex with FOXO3a and Nuclear respiratory factor (NRF)1 at the SIRT6 promoter to positively regulate SIRT6 expression (Kim et al. 2010).

5.2.2 Post-translational Modification

As previously stated, the other major mechanism for regulating Sirtuin function is PTM, which can occur at different levels. For instance, in response to oxidative stress, JNK kinase phosphorylates and stimulates SIRT1. In contrast, under stress, mTOR phosphorylates and inhibits SIRT1, suggesting a complex regulatory mechanism that could temporally regulate SIRT1 activity (Back et al. 2011; Nasrin et al. 2009). Moreover, under stress different kinases phosphorylate SIRT1 at different residues, modifying its p53 deacetylation capacity. For example, phosphorylation of SIRT1 by DYRK1A or DYRK3 stimulates it to deacetylate p53 (Guo et al. 2010), and by AMPK, inhibits its capacity to deacetylate p53 (Lee et al. 2012). SIRT1 undergoes other PTMs besides phosphorylation. For instance, in response to DNA damage, it is methylated by Set7/9, which inhibits its ability to deacetylate p53 (Liu et al. 2011). Alternatively, under genotoxic stress, sumoylation of SIRT1 by SUMO1 increases its activity, whereas desumoylation of SIRT1 by SENP1 reduces its catalytic activity (Yang et al. 2007).

SIRT2, SIRT6 and SIRT7 are also modified by phosphorylation, mainly during mitosis. The roles of these modifications (e.g. SIRT2 P-S368 or SIRT6 P-S303) are poorly understood, although they appear to relate to mitotic progression. In the case of SIRT6, phosphorylation of residue S338 has been suggested to participate in protein interaction (Dephoure et al. 2008; Miteva and Cristea 2014; North and Verdin 2007). Finally, another interesting modification in SIRT6 is its auto ADP-ribosylation, although its role also is unknown (Liszt et al. 2005).

5.3 Sirtuins in Stress Response

As previously mentioned, Sirtuins coordinate stress response at many levels, which can be divided into four groups: *genome stability*, *regulation of gene expression*, *metabolism* and *apoptosis and senescence*.

5.3.1 Genome Stability

Maintenance of genome integrity is essential for preventing tumorigenesis and ensuring cell viability. Sirtuins regulate genome stability via three principal processes: by *regulating chromatin*, through modulation of its structure and dynamics; by *participating in DNA repair* pathways; and finally, by *regulating cell-cycle progression*, which apparently includes direct involvement in checkpoint regulation (Fig. 5.1).

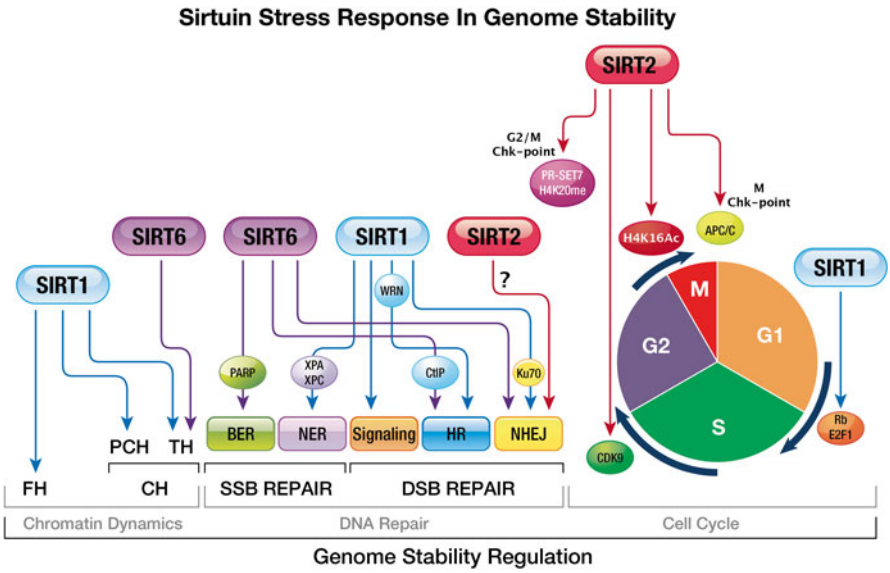


Fig. 5.1 Response of Sirtuins to stress: ensuring genome stability. Sirtuins are keystones of cellular stress response, regulating genome stability via three main cellular processes: *chromatin dynamics DNA repair* and *cell-cycle progression*. **Chromatin dynamics**: SIRT1 and SIRT6 are the only Sirtuins that regulate heterochromatin formation. Under stress, SIRT1 promotes formation of FH and CH at pericentromeric and telomeric regions. However, SIRT6 only promotes formation of TH. **DNA repair** (SSB and DSB): Sirtuins have been imputed in DNA-repair signaling and response. SIRT6 activates BER by mono(ADP-ribosyl)ating PARP1 and SIRT1 activates NER via up-regulation of the repair enzymes XPA and XPC. SIRT1, SIRT6 and SIRT2 are important for DSB repair. In HR SIRT6 promotes DNA-end resection by regulating CtIP and SIRT1 activates the helicase WRN. SIRT1 induces NHEJ by deacetylating Ku70, thereby inducing Ku70-dependent DNA repair. **Cell-cycle progression**: the main Sirtuin involved in cell-cycle regulation is SIRT2. During the G2/M transition, SIRT2 deacetylates H4K16Ac and the HMT PRSET7, enabling deposition of H4K20me1 by the latter. In metaphase, SIRT2 activates APC/C, enabling mitotic exit. SIRT1 promotes cell proliferation by regulating Rb/E2F1. Abbreviations: *BER* base excision repair, *Chk-point* checkpoint, *CH* constitutive heterochromatin, *FH* facultative heterochromatin, *HR* homologous recombination, *NER* nucleotide excision repair, *NHEJ* non-homologous end joining, *PCH* pericentromeric heterochromatin, *TH* telomeric heterochromatin

5.3.1.1 Regulation of Chromatin Structure and Dynamics

Regulation of chromatin structure is among the most important of the stress responses mediated by Sirtuins. However, only SIRT1 and SIRT6 are known to have this function, which they perform by deacetylating conserved histone marks and by modulating other chromatin factors, including transcription factors and chromatin enzymes.

Facultative Heterochromatin (FH)

In response to stress, SIRT1 acts as an expression silencer: it promotes FH formation, which results in silencing of specific genes linked to specific pathways in the context of stress response (see below). In fact, SIRT1 promotes epigenetic silencing of the targets regulated by major transcription factors, by coordinating several events together with other enzymes (Vaquero et al. 2007). Chromatin immunoprecipitation experiments have shown that when SIRT1 is recruited to a euchromatin region, there is a reduction in the euchromatin marks H3K79me2, H4K16Ac and H3K9Ac; recruitment and deacetylation of histone H1; and an increase and spreading of heterochromatin marks such as H3K9me3 and H4K20me1 (Vaquero et al. 2004). In this sense, SIRT1 promotes the spreading of the repressive mark H3K9me3, through its functional relationship with the histone methyltransferase (HMT) SUV39h1, the main H3K9me3 activity in mammals. SIRT1 interacts with, recruits and deacetylates SUV39h1 at its catalytic domain, thereby increasing SUV39h1 activity (Vaquero et al. 2007).

Another example of SIRT1 in FH occurs during development. SIRT1 levels are high in non-differentiated cells and decrease with increasing differentiation. This is interesting in the development of tissue types that are particularly sensitive to energy or redox fluctuations, such as brain tissue. SIRT1 is part of Polycomb Repressive Complex 4 (PRC4), which contains the H3K27me3 HMT Ezh2 (Kuzmichev et al. 2002). PRC4 activity results in methylation of H1K26 through previous deacetylation of H1K26 by SIRT1. This in turn would create a binding site for heterochromatin protein (HP) 1, thereby promoting heterochromatinization (Vaquero 2009). Consistently with this scenario, all subunits of the PRC complexes, including SIRT1, are overexpressed in breast, colon and prostate cancers (Chang and Hung 2012). SIRT1 also interacts with the histone H3K4 demethylase LSD1/KDM1A complex (Mulligan et al. 2011). This complex helps repress genes governed by the Notch signaling pathway, via H4K16Ac deacetylation and H3K4me1/2 demethylation. Said pathway is critical in mammalian development and is involved in tumorigenesis (Mulligan et al. 2011).

Another important example of SIRT1 in FH is the regulation of ribosomal DNA (rDNA) repression in the nucleolus. Due to its highly repetitive nature, the rDNA locus is a strong candidate for homologous recombination events, leading to unwanted chromosomal rearrangements. Nucleolar SIRT1, SUV39h1 and the H3K9me2-binding protein nucleomethylin are all components of the e-NoSc

complex, which silences the rDNA locus by controlling ribosome biosynthesis under nutrient or energy scarcity (Murayama et al. 2008). Therefore, this complex provides a regulatory link between cellular energy balance and the epigenetic state of the rDNA locus (Murayama et al. 2008).

Constitutive Heterochromatin (CH)

Constitutive heterochromatin are always maintained as heterochromatin, play a structural role in the physical organization of the genome in the nucleus, contain few genes and are located primarily in pericentromeric regions and telomeres. By maintaining the structure of CH, Sirtuins play a crucial role in genomic stability. To date, only SIRT1 and SIRT6 have been directly linked to CH.

SIRT1 is the main Sirtuin involved. It has been related to pericentromeric heterochromatin (PCH) and telomere regulation (Vaquero et al. 2007). Indeed, loss of SIRT1 in mice has been associated with a loss of PCH foci and with derepression of the underlying γ-satellites (Bosch-Presegué et al. 2011). Involvement of SIRT1 in CH is directly related to its functional relationship with SUV39h1, a keystone of CH formation (Peters et al. 2001): loss of SIRT1 impairs SUV39h1-dependent H3K9me3 and HP1 localization in PCH (Vaquero et al. 2007). Given the limited amount or even absence of SIRT1 in PCH foci, one possible explanation for this functional loss is that SIRT1 might regulate PCH structure by preventing proteasomal degradation of SUV39H1. *In vivo* this increase in the levels of SUV39h1 results in its fast turnover in pericentromeric heterochromatin regions, which contributes to genome protection. Thus, stress leads to SIRT1-dependent upregulation of SUV39h1 *in vivo*, suggesting a direct link between the stress response and SUV39h1 dynamics in heterochromatin structure as a mechanism of genome stability (Bosch-Presegué et al. 2011).

The role of SIRT1 in telomeres is controversial. Some evidence suggests that SIRT1 inhibits telomerase activity by regulating the stability of the telomerase reverse transcriptase (TERT) catalytic subunit (Narala et al. 2008). In contrast, a battery of other findings suggests the opposite. For instance, $Sirt1^{-/-}$ cells exhibit reduced genome stability as well as telomeric aberrations that contribute to decreased cell growth (El Ramy et al. 2009). Furthermore, silencing of SIRT1 results in reduced expression of the shelterin complex components TERT and PTOP (Chen et al. 2011). Moreover, SIRT1 interacts with murine telomeric repeats, thereby attenuating the telomere shortening associated with ageing. Finally, $SIRT1^{super}$ mice, which contain an extra copy of the SIRT1 gene, show improved telomere maintenance owing to telomerase activation (Palacios et al. 2010).

SIRT6 also has been linked to telomere integrity maintenance, as it is critical for preventing telomere dysfunction and aberrant chromosomal end-to-end fusions. Deacetylation of H3K9Ac and H3K56Ac by SIRT6 in telomeres is required for efficient binding of helicase WRN to telomeres during the S-phase, to ensure efficient telomere replication and to prevent accumulation of structural abnormalities at telomeres (Michishita et al. 2008, 2009; Yang et al. 2009). Considering that H3K9Ac

and H3K56Ac are also targets of SIRT1 and SIRT2, there might be some functional redundancy among Sirtuins in this context. Additionally, SIRT6 regulates telomere-proximal transgene silencing and has been linked to telomere position effect variegation (Tennen et al. 2011).

5.3.1.2 DNA Damage Repair: Signaling and Response

Another important role for Sirtuins is in DNA repair. Sirtuins participate in the signaling and repair of single-strand breaks (SSBs) and double-strand breaks (DSBs) at different levels. To date, the Sirtuins known to have the greatest involvement in DNA repair are SIRT1 and SIRT6.

DNA SSB

Sirt6$^{-/-}$ mice suffer from increased radiation sensitivity, chromosomal aberrations and impaired DNA repair. They also exhibit a premature aging phenotype associated with impaired base excision repair (BER), the main repair pathway for SSBs. However, a role for SIRT6 in BER remains unknown, because SIRT6 does not appear to interact or co-localize with BER factors at the damage sites (Mostoslavsky et al. 2006). Nonetheless, SIRT6 seems to indirectly regulate BER by modulating chromatin density and accessibility to DNA damage sites (Jia et al. 2012). Under oxidative stress conditions, SIRT6 is recruited to DNA damage sites, where it stimulates repair through direct interaction and ADP-ribosylation of PARP1, an enzyme involved in BER and DSB signaling. The role of SIRT6 as PARP1 activator might explain the deficiencies in BER and in genomic stability related to SIRT6 depletion (Mao et al. 2011).

SIRT1 has been linked to the nucleotide excision repair (NER) pathway for the repair of SSBs. Deacetylation of xeroderma pigmentosum complementation group A (XPA) by SIRT1 increases its interaction with RPA32, an essential event for the NER pathway (Fan and Luo 2010). SIRT1 also regulates the expression of XPC by reducing AKT-dependent nuclear localization of its transcriptional repressor (Ming et al. 2010).

DNA DSB

SIRT1, SIRT6 and, to a lesser extent, SIRT2, are involved in DSB repair at different levels. Upon γ-irradiation, SIRT1 plays a crucial role in the very early stages of DSB signaling, by modulating the formation of γ-H2AX, BRCA1, Rad51 and NBS1 foci (Wang et al. 2008). SIRT1-dependent deacetylation of NBS1, a regulatory subunit of the MRE11-Rad51-NBS (MRN) complex, modulates its activity as an intra-S phase checkpoint factor. Furthermore, relocalization of SIRT1 to DSBs depends on ATM-mediated signaling through H2AX phosphorylation (Oberdoerffer

et al. 2008). SIRT1 also participates in DSB signaling through hMOF and TIP60, two members of the MYST family of histone acetyltransferases that are involved in cell growth (and growth arrest), apoptosis and DNA repair. Under normal conditions, SIRT1 binds to and deacetylates hMOF and TIP60, inhibiting their activities and promoting their ubiquitination-dependent degradation. However, DNA damage results in decreased binding of SIRT1 to both hMOF and TIP60 and activates DNA-damage signaling (Peng et al. 2012; Sun et al. 2009).

Interestingly, both SIRT1 and SIRT6 are directly involved in the two major DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). SIRT6 apparently plays an important role in HR, promoting DNA-end resection through interaction with, and deacetylation of, C-terminal binding protein interacting protein (CtIP) (Kaidi et al. 2010). CtIP is constitutively acetylated, but upon DNA damage, it is deacetylated by SIRT6 to promote resection together with Breast cancer 1 (BRCA1). Accordingly, SIRT6 loss results in reduced rates of HR and in sensitization of cells to DSB-inducing agents. Interestingly, a decrease in HR observed in replicative aging can be restored by SIRT6 overexpression. SIRT6 also interacts with factors of the NHEJ pathway, including DNA-PKcs and Ku70/80. SIRT6 depletion strongly diminishes recruitment of DNA repair factors *in vitro* and *in vivo* (Toiber et al. 2013). Accordantly, SirT6 is required for changes in chromatin structure surrounding DSBs, as it promotes a decrease in H3K9Ac levels to stabilize the association of DNA-PKcs to chromatin and to enable repair factors to access the DNA lesions (McCord et al. 2009). Additionally, deacetylation of H3K56 by SIRT6, and interaction of SIRT6 with the ATP-dependent chromatin remodeler SNF2H, are required for efficient DSB repair.

SIRT1 is linked to HR through its interaction and deacetylation of WRN helicase, a member of the RecQ family (Uhl et al. 2010). SIRT1 has been linked to NHEJ through deacetylation of the factor Ku70, which results in induction of Ku70-dependent DNA repair and inhibition of apoptosis through nuclear retention of the proapoptotic factor BAX (Cohen et al. 2004; Sawada et al. 2003).

Little is known about SIRT2 in DSB repair. SirT2 deacetylates H3K56Ac (Vempati et al. 2010) during S-phase and H4K16Ac during G_2/M (Vaquero et al. 2006). Both marks are involved in DNA damage-repair response and are cell-cycle-dependent. Under normal conditions, H3K56Ac is spread throughout the nucleus; however, upon DNA damage, its levels increase and it concentrates in DNA damage foci, where it colocalizes with γ-H2AX, pATM, Chk2 and p53 (Vempati et al. 2010). Contrariwise, deacetylation of H4K16Ac at DNA damage sites is required for recruitment of 53BP1 to DSBs and for ensuring NHEJ repair (Hsiao and Mizzen 2013). SIRT2 is also linked to DNA repair through its ability to promote deposition of H4K20me1 during G_2/M. Interestingly, establishment of H4K20me1 is also required for deposition of di- and tri- H4K20 methylation (H4K20me2/3) throughout the cell cycle. H4K20me1 levels indirectly affects DNA damage repair, since H4K20me2 is required for recruitment of 53BP1 to the DNA damage foci (Hartlerode et al. 2012; Serrano et al. 2013). However, some studies suggest that a portion of the H4K20me2 is not derived from the mitotic H4K20me1 and is deposited *de novo* in S-phase at DSB foci by the HMT MMSET (Pei et al. 2011).

5.3.1.3 Cell-Cycle Control

Regulation of cell-cycle progression is another mechanism for maintaining genome integrity. By regulating cell-cycle arrest, cells provide the DNA repair machinery with sufficient time to fix damage generated during the cycle or, in extreme cases, to activate apoptotic pathways. The Sirtuins most involved in cell-cycle control are SIRT2 and, to a lesser extent, SIRT1.

The link between SIRT2 and genome stability is well defined, as evidenced by the finding that $Sirt2^{-/-}$ mice exhibit major genome instability and are prone to develop tumors. Several studies support the role of SIRT2-deacetylase activity in the control of cell-cycle progression. SIRT2 is shuttled from the cytoplasm to the nucleus during the G_2/M transition (Vaquero et al. 2006), where it is phosphorylated at S368 by CDK1. At the end of mitosis, CDC14A and CDC14B dephosphorylate SIRT2, thereby promoting its degradation and enabling mitotic exit (North and Verdin 2007). In fact, some evidence suggests that before being degraded, SIRT2 might also be involved in mitotic exit (Dryden et al. 2003). During the G_2/M transition, SIRT2 controls genome stability by modulating chromatin condensation through deacetylation of H4K16Ac and regulation of H4K20me1 deposition. Thus, SIRT2 regulates the activity and the chromatin binding of the H4K20me1 HMT PR-SET7 and has a crucial role in spreading of H4K20me1 throughout the chromosome, thereby ensuring correct packing of chromatin during G_2/M. Thus, SIRT2-dependent deacetylation of PR-SET7 increases its stability in chromatin and its catalytic activity towards H4K20. This regulation is crucial for the G_2/M transition and is directly involved in G_2/M checkpoint control. Under stress during G_2/M, SirT2 binds strongly to PR-SET7, leading to an increased level of H4K20me1 and preventing cells from entering mitosis (Serrano et al. 2013). SirT2 is also related to the metaphase/anaphase checkpoint—probably via regulation of centrosome replication and modulation of Anaphase Promoting Complex/Cyclosome (APC/C) activity. Thus, SirT2 is partially localized in centrosomes (Kim et al. 2011), where it interacts with Aurora A, an essential protein for centrosome replication (Cowley et al. 2009). SirT2 deacetylates CDH1 and CDC20, two E3-ligases of the APC/C complex, which is essential for mitotic progression, which positively regulates its activity. After SirT2-dependent deacetylation, CDH1 and CDC20 bind APC/C complex, promoting Aurora-degradation, preventing abnormalities in centrosome replication and enabling mitotic exit (Kim et al. 2011). SIRT2 is also required for S-phase progression after replication stress. Stalled replication forks containing ssDNA activate the replication stress response (RSR), which involves cell-cycle arrest, and stabilization and recovery of the stalled replication forks, to maintain genome stability. In this context, SIRT2 deacetylates and activates CDK9, a kinase required for the replication stress response. Accordingly, cells lacking either SIRT2 or CDK9 exhibit delayed S-phase progression after replication stress, due to impaired recovery from transient replication arrest (Zhang et al. 2013).

The role of SIRT1 in the cell cycle is related to the G_1/S transition and to mitosis. In the first case, SIRT1 regulates the G_1/S factors Retinoblastoma (Rb) and E2F1. Rb is a tumor suppressor that controls the G_1/S transition by binding to

E2F-responsive genes. SIRT1 deacetylates Rb and promotes its phosphorylation, thereby inhibiting Rb-dependent apoptosis and promoting cell proliferation (Wong and Weber 2007). Moreover, SIRT1 can also regulate the transcriptional repressive activity of the HDAC1-containing Rbp1 complex, thereby inhibiting its growth-arrest activity (Binda et al. 2008). In the second case, SIRT1 deacetylates histones and participates in loading of the Condensin I complex and H1 in chromatin, thereby facilitating chromosome condensation. SIRT1 depletion disrupts this loading and results in improper chromatin condensation and aberrant mitosis (Fatoba and Okorokov 2011).

5.3.2 Regulation of Gene Expression

As previously mentioned, an important function of Sirtuins in stress response is their role in regulating the stress-response expression programs of numerous transcription factors, including NF-κB, p53, HIF-1α, FOXOs, E2F1, PGC-1α and HSF1 (Fulco et al. 2003; Luo et al. 2001; Muth et al. 2001; Senawong et al. 2003; Takata and Ishikawa 2003; Vaziri et al. 2001). The Sirtuins most clearly involved in these processes are SIRT1, SIRT6 and, to a lesser extent, SIRT7.

Among the most important of the aforementioned transcription factors are the members of the FOXO family. FOXOs are involved in controlling the G_1/S and G_2/M checkpoints, and in inducing expression of genes involved in DNA-damage response, differentiation, glucose metabolism, and apoptosis (Huang and Tindall 2007). Under oxidative stress, Sirtuins target FOXO transcription factors, thereby dictating their subcellular localization, protein stability, and transcriptional activity (Brunet et al. 2004). Acetylation of FOXOs reduce their DNA binding and enhance their phosphorylation and inactivation. Deacetylation of FOXO by SIRT1 alters its interactions with E2F1 or p53, leading to apoptosis or to cell-cycle arrest, depending on the cellular metabolic state, environmental conditions, and tissue. Under oxidative stress, SIRT1 deacetylates FOXO3a, thereby increasing its capacity to promote cell-cycle arrest and preventing the cell from inducing apoptosis. Furthermore, SIRT1 enhances the cellular defense response to oxidative stress, by deacetylating and activating FOXO1 and FOXO4, inducing the expression of the cyclin/cdk inhibitor $p27^{Kip1}$, manganese superoxide dismutase (MnSOD) and Growth arrest and DNA damage-inducible protein (GADD45) (Brunet et al. 2004; Daitoku et al. 2004; van der Horst and Burgering 2007). Analogously to SIRT1, SIRT2 deacetylates FOXO3a, enhancing its DNA binding and promoting expression of FOXO target genes, $p27^{Kip1}$, MnSOD and the pro-apoptotic protein Bim. Consequently, SIRT2 promotes cell-cycle arrest, reduces cellular levels of ROS and, under severe stress, triggers apoptosis (Wang et al. 2007) (Fig. 5.2a).

Another important transcription factor is nuclear factor-κB (NF-κB), which is vital for regulating expression of certain genes involved in aging, proliferation and inflammation. Different subunits of the NF-κB family are acetylated and deacetylated at multiple sites, affecting its DNA-binding and transcriptional activity, and

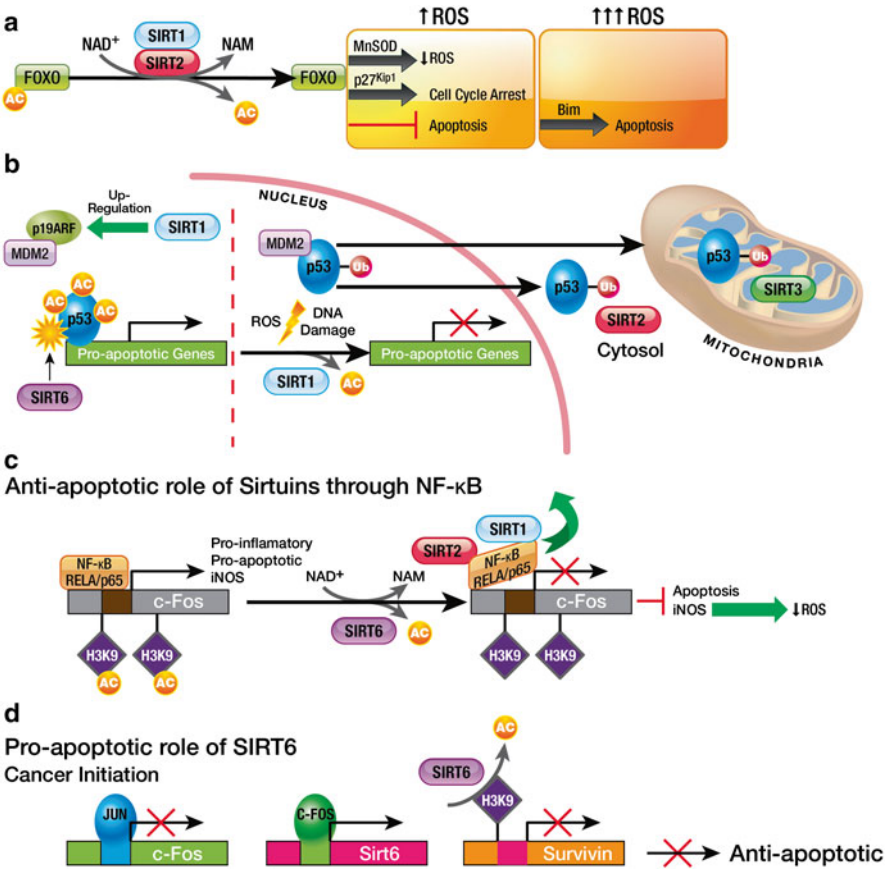


Fig. 5.2 Sirtuins in apoptotic pathways. (**a**) **SIRT1 and SIRT2-dependent regulation of FOXO under oxidative stress**. Under mild oxidative stress, SIRT1 and SIRT2 promote reduced ROS production and cell-cycle arrest, by activating the FOXO target genes MnSOD and $p27^{Kip1}$, respectively. Under severe stress, this regulation triggers apoptosis. (**b**) **Sirtuin-dependent regulation of p53**. SIRT1 seems to regulate replicative life span by up-regulating $p19^{ARF}$, consequently decreasing MDM2-dependent degradation of p53. Under stress, SIRT1 deacetylates p53, thereby promoting its monoubiquitination and its shuttling from the nucleus to the cytosol and mitochondria. Depending on the cellular state, SIRT1 either promotes or inhibits apoptosis through p53. SIRT6 activates p53 by mono[ADP-ribosyl]ating it. (**c**) **Anti-apoptotic role of Sirtuins in pro-inflammatory pathways**. SIRT6 is recruited by the NF-κB subunit RELA/p65 to pro-inflammatory genes, where it blocks NF-κB signaling by deacetylating H3K9Ac. In this context, SIRT1 and SIRT2 bind to NF-κB, removing it from chromatin and thereby contributing to the end of the pro-inflammatory signal. (**d**) Role of SIRT6 in oncogenesis: deregulation of the c-JUN pathway. SIRT6 represses expression of the anti-apoptotic gene survivin by deacetylating H3KAc in its promoter. Expression of Sirt6 is promoted by c-FOS, whose expression is repressed by c-JUN. During oncogenesis, c-JUN inhibits expression of c-fos and consequently, blocks repression of survivin, thereby leaving the cell to proliferate

consequently, modulating the release of pro-inflammatory mediators. In this sense, SIRT6 plays an anti-inflammatory role, by regulating the NF-κB pathway and decreasing NF-κB-dependent apoptosis and senescence. Following activation of NF-κB by Tumor necrosis factor alpha (TNF-α), SirT6 interacts with the NF-κB subunit RELA/p65 and is recruited to promoters of a subset of NF-κB target genes. Subsequently, SIRT6 deacetylates H3K9Ac at those promoters and destabilizes RELA-promoter interaction, thereby contributing to NF-κB signal termination (Kawahara et al. 2009) (Fig. 5.2c). However, SIRT6 is not the only Sirtuin that modulates this pathway: SIRT1 and SIRT2 each interact with and deacetylate the RELA/p65 subunit, which results in NF-κB-mediated transcription inhibition (Rothgiesser et al. 2010; Oeckinghaus and Ghosh 2009). This effect in turn suppresses induction of nitric oxide synthase (iNOS) and nitrous oxide production, and might reduce the amount of cellular ROS (Lee et al. 2009). Additionally, treatment of cells with Resveratrol, a SIRT1 activator, correlates to a loss of expression of NF-κB-regulated genes and to sensitization of cells to TNFα-induced apoptosis. These findings suggest that SIRT1 activity, via NF-κB inhibition, augments apoptosis in response to TNFα (Yeung et al. 2004).

Interestingly, another anti-inflammatory role for SIRT6 has been described in $Sirt6^{-/-}$ mice. This role involves the transcription factors c-JUN and c-FOS, which are members of the AP-1 signal-transducing family and are implicated in cell-cycle progression, cell differentiation and apoptosis. c-FOS regulates SIRT6 expression. In turn, SIRT6 interacts with c-JUN and deacetylates H3K9Ac at promoters of pro-inflammatory genes including TNFα, MCP-1 and IL-6 (Xiao et al. 2012). Accordingly, SIRT6 represses expression of the anti-apoptotic protein survivin by H3K9Ac deacetylation. During liver tumor initiation, c-JUN regulates survival of initiated cancer cells through repression of c-fos transcription, which in turn down-regulates SIRT6 expression, enabling expression of survivin and tumor initiation (Fig. 5.2d). In fact, overexpression of SIRT6 during liver tumor initiation is sufficient to decrease subsequent liver tumorigenesis (Min et al. 2012).

Stress affects more than just regulation of protein-coding genes: genotoxic stress, CR and other types of stress can also alter transcription of ribosomal RNA (rRNA). RNA-polymerase I (Pol-I) transcription has been shown to be repressed by nucleolar SIRT1, via deacetylation of its basal component TAF_I68 (Ford et al. 2006). Interestingly, SIRT7 is a component of the Pol-I transcriptional machinery and enhances its transcriptional activity, performing an antagonistic role to that of SIRT1. SIRT7 depletion results in decreased association of Pol I with rDNA and consequently, in reduced Pol-I transcription. During mitosis, SIRT7 is phosphorylated and remains localized to the nucleolus until telophase, when it is activated (by dephosphorylation) to help rDNA expression resume after mitosis. SIRT7 has also been linked to repress transcription of a set of non-nucleolar genes via deacetylation of H3K18Ac at their promoters. SIRT7 deacetylates H3K18Ac at promoters of a network of genes linked to tumor suppression through its interaction with the cancer associated transcription factor ELK4. This SIRT7-associated function is related to the maintenance of an oncogenic phenotype in transformed cells, including anchorage-independent growth and escape from contact inhibition, which

suggests an important role for SIRT7 as a tumorigenic factor (Barber et al. 2012). Another major category of genes targeted by SIRT7 is the ribosomal protein genes, which are transcriptional targets of the oncogenic protein MYC. In this context, SIRT7 plays an adaptive role in the Unfolded Protein Response (UPR) to suppress endoplasmic reticulum (ER) stress. During ER stress, cells must stop protein biosynthesis. Under these conditions, SIRT7 is up-regulated and is stabilized by MYC to the promoters of ribosomal protein genes, repressing its transcription and preventing the cell from initiating new protein biosynthesis. During hypoxia, cancer cells must decrease their energy expenditure and focus on survival. To this end, SIRT7 reduces expression of ribosomal protein genes, thereby enabling tumor progression (Shin et al. 2013).

5.3.3 Metabolic Energetic Mechanisms

Among the most important and conserved roles of Sirtuins is to modulate metabolic adaptation to different types of stress, as evidenced by the fact that three Sirtuins (SIRT3, SIRT4 and SIRT5) are localized to the mitochondria, where they control mitochondrial energy production, substrate oxidation and apoptosis (Onyango et al. 2002; Jeong et al. 2013).

Sirtuins have been linked to CR, which extends lifespan and reduces ROS production by shifting cell metabolism from glycolysis to fatty acid oxidation, amino acid metabolism and ketogenesis (Fig. 5.3). The mitochondrial Sirtuins, together with SIRT1 and SIRT6, apparently coordinate this metabolic shift—a function that would intertwine them with CR, aging and carcinogenesis (Koubova and Guarente 2003; Qiu et al. 2010). For example, under CR, SIRT3 is up-regulated (Han and Someya 2013) and in turn, deacetylates acetyl-CoA synthetase 2 (AceCS2). AceCS2 is expressed in muscle and converts acetate into acetyl-CoA under ketogenic conditions such as CR, thereby enabling muscle to use acetate as a carbon source (Schwer et al. 2006).

Among the functional connections between Sirtuins and metabolism is regulation of mitochondrial targets by acetylation/deacetylation. Most mitochondrial proteins are acetylated, including the major regulators of mitochondrial metabolism, and all three mitochondrial Sirtuins have been shown to deacetylate mitochondrial targets (Lombard et al. 2007; Haigis et al. 2012; Nakagawa and Guarente 2009). Among mitochondrial deacetylases, SIRT3 seems to predominate, as indicated by the finding that mitochondrial proteins are massively acetylated in $Sirt3^{-/-}$ mice. However, a small pool of SIRT3 can also be found in the nucleus under normal conditions, where it deacetylates H4K16Ac and H4K56Ac, which are essential marks in DNA damage response and in chromatin condensation (Scher et al. 2007; Vempati et al. 2010).

SIRT3 also seems to have an important role in ROS detoxification at different levels. Firstly, activation of SIRT3 might help increase mitochondrial respiration and ATP production, as indicated by the finding that SIRT3 deacetylates and

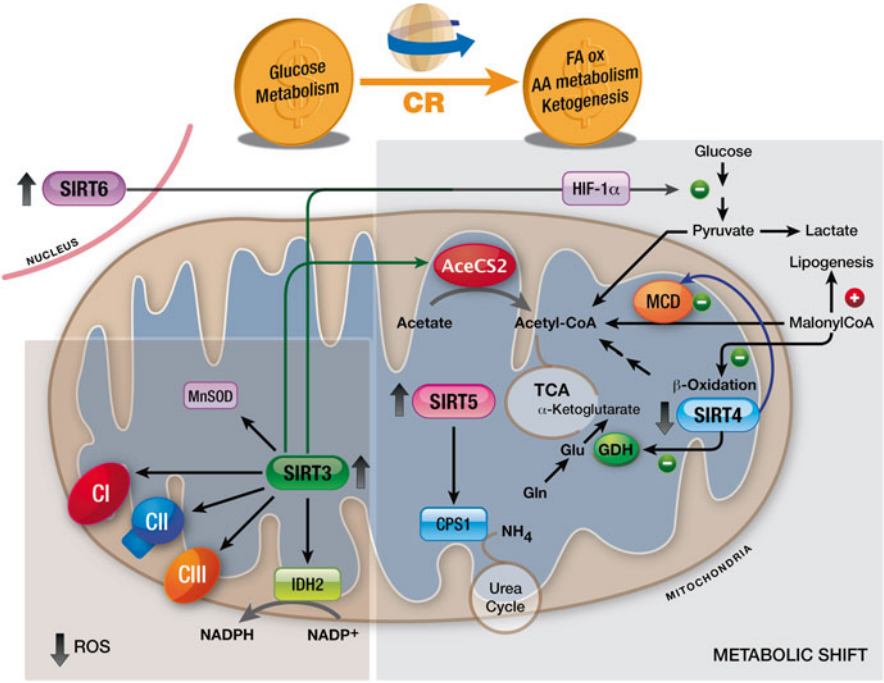


Fig. 5.3 Mitochondrial Sirtuins and SIRT6 during CR. CR shifts cell metabolism from glycolysis to FA oxidation, AA metabolism and ketogenesis. Increased activity of SIRT3 promotes reduced production of ROS by activating IDH2, the electron transport chain and MnSOD. Moreover, SIRT3, SIRT4, SIRT5 and SIRT6 drive the metabolic shift triggered by CR. Abbreviations: *AA* amino acid, *CR* calorie restriction, *FA* fatty acid, *TCA* tricarboxylic acid

activates complex I-III of the electron transport chain, thereby ensuring proper reduction of oxygen and avoiding ROS production (Cimen et al. 2010). Secondly, under CR, SIRT3 activates isocitrate dehydrogenase 2 (IDH2), an enzyme responsible for the conversion of $NADP^+$ to NADPH in mitochondria (Someya et al. 2010). Thirdly, by deacetylating the transcription factor FOXO3a, SIRT3 regulates the expression of manganese superoxide dismutase (MnSOD), an enzyme responsible for superoxide detoxification (Kim et al. 2010). Additionally, SIRT3 deacetylates MnSODK122, thereby increasing its enzymatic detoxification activity (Tao et al. 2010). Altogether, regulation of the electron transport chain, and of MnSOD activity and expression, by SIRT3 maintains the cellular balance of ROS. Accordingly, upon exposure to ionizing radiation, $Sirt3^{-/-}$ MEFs exhibit increased levels of superoxide, decreased stress-induced apoptosis, infrequent contact inhibition and increased chromosome instability (Sundaresan et al. 2009; Kim et al. 2010).

Evidence suggests that SIRT3 and SIRT6 each act as tumor suppressor by regulating cancer cell metabolism. Loss of SIRT3/SIRT6-dependent metabolic regulation has been directly associated with a metabolic switch to anaerobic glycolysis, known as the *Warburg effect*, which is considered a hallmark of cancer cells

(Kim et al. 2010; Zhong et al. 2010). This switch leads to a situation very similar to hypoxia. The major regulatory factor of hypoxia is HIF-1, which comprises a heterodimer of HIF-1α and HIF-1β. During normoxia, HIF-1α is hydroxylated by a family of oxygen-dependent prolyl hydroxylases (PHD1-3), flagging HIF-1α for ubiquitination and subsequent proteasomal degradation. However, under hypoxia, HIF-1α is stabilized and promotes expression of certain HIF-1α dependent pro-proliferative and pro-survival genes. Hypoxia also triggers an increase in ROS production, which inhibits PHD and consequently, stabilizes HIF-1α. Interestingly, the mechanism by which SIRT3 regulates glycolysis involves HIF-1α and its target genes: SIRT3 maintains ROS equilibrium, thereby preventing inactivation of PHD and keeping HIF-1α levels low (Bell et al. 2011). Likewise, SIRT6 controls glucose homeostasis by inhibiting several glycolytic genes (by deacetylating H3K9Ac), including many that are targets of HIF-1α. Accordingly, Sirt6$^{-/-}$ mice present hyperacetylation of H3K9 at these promoters and increased gene expression, which results in increased glucose uptake and glycolysis, boosting cell growth and proliferation (Zhong et al. 2010). SIRT6 has also been found to corepress MYC transcriptional activity towards ribosomal genes. Therefore, SIRT6 acts as an important tumor suppressor, modulating glycolysis and inhibiting ribosome biosynthesis, by corepressing HIF-1α and MYC transcriptional activity.

Interestingly, SIRT3 and SIRT6 are not the only players in the Warburg effect: SIRT7 and SIRT1 are also involved, although the contribution of the latter is under discussion. Evidence suggests that SIRT7 regulates genes related to adaptation of cancer cells to hypoxia, through HIF-1α and HIF-2α. However, the effect of SIRT7 does not depend on its activity, and SirT7 knockdown cells show upregulation of HIF-1α and HIF-2α (Hubbi et al. 2013). Regarding SIRT1, some results indicate that SIRT1-mediated deacetylation of HIF-1*α* represses its transcriptional activity, whereas others suggest that under hypoxia, SIRT1 stabilizes HIF-1*α*, similarly to the way it regulates HIF-2*α* under the same conditions (Lim et al. 2010; Laemmle et al. 2012; Dioum et al. 2009).

SIRT4 has a crucial role in regulating amino acid metabolism. Under normal conditions, glutamine is metabolized to glutamate and NH_4^+ by glutaminase, and then the glutamate is transformed to α-ketoglutarate, an intermediate of the TCA cycle, by glutamate dehydrogenase (GDH). Interestingly, following DNA damage, glutamine anaplerosis is reduced. Glutamine is essential for cellular proliferation and is required for the G_1/S transition; thus, the upregulation of SIRT4 upon DNA damage, and the subsequent decrease in glutamine usage, provoke a SIRT4-dependent arrest in the cell cycle. Consistently with this role for SIRT4 in the cell cycle, Sirt4$^{-/-}$ cells exhibit greater chromosome instability resulting from improper DNA damage-repair response. Consistently, SIRT4 ADP-ribosylates and inhibits the activity of GDH, thereby repressing glutamine anaplerosis in response to DNA damage (Jeong et al. 2013). However, under CR, SIRT4 expression is decreased, resulting in activation of GDH and subsequent increase of ATP production from glutamine (Haigis et al. 2008). Therefore, SIRT4 mitigates genomic instability and suppresses tumorigenesis by inhibiting glutamine metabolism (Jeong et al. 2013). Another functional link between SIRT4 and tumor suppression is the ability to

decrease the proliferation rate of Myc-induced B lymphoma cells by inhibiting mitochondrial glutamine metabolism (Jeong et al. 2014). Furthermore, SIRT4 promotes lypogenesis and inhibits lipid oxidation, by regulating MalonylCoA decarboxylase (MCD), an enzyme that generates acetyl-CoA from malonyl-CoA. MalonylCoA provides the carbon skeleton for lipogenesis and in turn, inhibits lipid oxidation. Under high nutrient availability, SIRT4 is active, and it inactivates MCD by deacetylation, thereby increasing levels of MalonylCoA and consequently, promoting lipogenesis (Laurent et al. 2013).

Although SIRT5 was originally described as a deacetylase, recent evidence supports a broader activity for it: as a lysine deacylase that can remove acetyl groups as well as malonyl, succinyl and other groups (Peng et al. 2011). For instance, SIRT5 desuccinylates and activates Carbamyl phosphatase synthetase 1 (CPS1), a critical enzyme in the urea cycle (Du et al. 2011). Consistently, SIRT5-deficient mice cannot cope with conditions of increased amino acid catabolism, such as CR (Nakagawa and Guarente 2009). SIRT5 also desuccinylates and activates SOD1, thereby reducing cellular ROS levels. This function is important in the context of lung cancer, as indicated by the finding that mutations in the succinylated lysine of SOD1 inhibit the growth of lung tumor cells (Lin et al. 2013).

5.3.4 Apoptosis and Senescence

Sirtuins also help regulate the p53 pathway: they and p53 interact at various levels to induce cell-cycle progression, senescence or apoptosis, depending on the cellular status of p53 (Yi and Luo 2010). Hyperacetylation of p53 enhances its activity, leading to increased expression of its pro-apoptotic targets and preventing its degradation (by inhibiting its interaction with MDM2). SIRT1-dependent deacetylation of p53 reduces its ability to induce expression of these targets, thereby suppressing apoptosis in response to DNA damage or to oxidative stress (Vaziri et al. 2001; Yi and Luo 2010) (Fig. 5.2b). Accordingly, $Sirt1^{-/-}$ mice exhibit higher levels of p53 hyperacetylation and higher levels of radiation-induced apoptosis compared to WT mice (Cheng et al. 2003). However, SIRT1 might regulate p53 in a more complex manner: for instance, by activating p53 via the p53-activator $p19^{ARF}$ (Chua et al. 2005). SIRT1 seems to limit replicative life span by regulating p53; however, under acute DNA damage, SIRT1 promotes DNA repair and survival by deacetylating p53 and inhibiting apoptosis.

SIRT1 also regulates subcellular localization of p53, determining its cellular fate under oxidative stress (Han et al. 2008). In mouse ESC cells, high levels of ROS induce SirT1–dependent accumulation of p53 in the cytosol and the mitochondria, thereby leading to transcription-independent p53-induced apoptosis. However, *in vivo* studies suggest that SIRT1 does not clearly affect p53-dependent functions: the hypersensitivity to radiation, and the apoptosis, observed in $Sirt1^{-/-}$ mice apparently do not depend on p53 activity (Kamel et al. 2006). This probably reflects a functional redundancy among Sirtuins. In fact, inhibition of both SIRT1 and SIRT2 in

cancer cells is required to sensitize them to p53-dependent apoptosis (Matsushita et al. 2005; Peck et al. 2010). SIRT3 deacetylates p53 in the mitochondria and rescues cells from p53-mediated cell arrest (Li et al. 2010). In addition, SIRT2 deacetylates p53 in the cytosol, thereby impairing its transcriptional activity (Jin et al. 2008). Additionally, SIRT7 deacetylates p53 *in vitro* and *in vivo*, and SIRT7 depletion by RNAi leads to inhibition of cell growth and to induction of p53-mediated apoptosis in U2OS cells and in primary cardiomyocytes (Vakhrusheva et al. 2008). Moreover, SIRT3 promotes survival of cardiomyocytes under stress: its binds to and deacetylates Ku70, thereby promoting the interaction between Ku70 and the pro-apoptotic protein BAX. This interaction impairs the translocation of BAX into the mitochondria and consequently, prevents apoptosis (Sundaresan et al. 2008; Marfe et al. 2009). Consistently, SIRT3 activates NF-κB, leading to increased expression of the anti-apoptotic protein BCL-2 and to decreased expression of BAX, thereby enabling the cell to survive (Chen et al. 2013). However, there is no consensus among researchers about the role of SIRT3 under stress; consequently, the role of SIRT3 in apoptosis under stress is poorly understood (Sundaresan et al. 2008).

5.4 Sirtuins and Cancer

Given that Sirtuins are linked to myriad pathways, they are double-edged swords in cancer, acting as either tumor suppressor or promoter according to the cellular or functional context (Table 5.2). Among Sirtuins, SIRT1 is the most studied in cancer regulation. Numerous substrates have been identified for SIRT1, which in turn is modulated by crucial regulators in cancer, cell proliferation, DNA damage repair, and survival under various stress conditions. Consequently, SIRT1 might play a dual role in cancer, depending on the tissue context and on the temporal and spatial distribution of factors upstream and downstream of it. The functioning of SIRT1 as tumor suppressor is corroborated by its role in maintaining genome stability through chromatin regulation and DNA repair. Accordingly, $Sirt1^{-/-}$ mouse embryos exhibit more chromosomal aberrations and impaired DNA repair than do WT embryos (Wang et al. 2008). Furthermore, $SIRT1^{super}$ mice show lower levels of DNA damage and decreased expression of the aging-associated gene p16 (Ink4a), and are partially protected from diabetes, osteoporosis and cancer. Additionally, $SIRT1^{super}$ mice are less prone to spontaneous carcinomas and sarcomas. Accordingly, in studies on a metabolic syndrome-associated liver cancer model, $SIRT1^{super}$ mice were less prone to liver cancer and exhibited greater hepatic protection from both DNA damage and metabolic damage compared to WT mice (Palacios et al. 2010).

Some evidence suggests that SIRT1 promotes tumorigenesis through its inhibitory role in senescence and apoptosis. Overexpression of SIRT1 can block stress-induced apoptosis via regulation of different pathways (e.g. p53, FOXO, E2F1, Rb, BCL6 and Ku70). Thus, Sirt1 is upregulated in various cancers; however, whether this is a *consequence* or a *cause* of cancer remains a matter of debate (Yuan et al. 2013). Interestingly, given the survival advantages provided by SIRT1, some tumors

Table 5.2 Sirtuins in cancer

			Altered in cancer	
	Role in cancer	KO mice cancer related phenotypes	Up-regulated	Down-regulated
SIRT1	Tumor suppressor/ oncogene	Chromosomal aberrations. Impaired DNA repair. Increased tumorigenesis	Chemoresistant leukemia, neuroblastomas, osteosarcomas, ovarian and breast cancer cell lines. Prostate cancer	Breast cancer, hepatic cell carcinomas
SIRT2	Tumor suppressor/ oncogene	Spontaneous gender-specific tumors in C57BBL/6-Black Swiss-FVB backgrounds: female: mammary glands male: liver, lung, pancreas, stomach, duodenum and prostate	MYC-induced malignances	Gilomas, esophageal adenocarcinomas, gastric adenocarcinomas, HNSCC
SIRT3	Tumor suppressor/ oncogene	ER-and PR-positive mammary invasive tumors	Lymph-node-positive breast cancer, OSCC	Glioblastoma, prostate, head and neck cancers
SIRT4	Tumor suppressor	Lung tumors	ND	Breast, colon, gastric, ovarian and thyroid carcinomas
SIRT5	ND	Non cancer related phenotypes defined	ND	ND
SIRT6	Tumor suppressor/ oncogene	Tumor formation and poor prognosis	Chronic lymphocytic leukemia, squamous cell carcinoma, paclitaxel-and epirubicin-resistant MCF-7 cells, breast cancer	Hepatocellular carcinoma, acute myeloid leukemia, head and neck squamous cell carcinoma, pancreatic cancer, colorectal cancer
SIRT7	Oncogene	Aging-like phenotype	Breast, thyroid and liver cancers	ND

ER estrogen, *HNSCC* head and neck squamous cell carcinoma, *KO* knock out, *ND* not determined, *OSCC* oral squamous cell carcinoma, *PR* progesterone

might become addicted to SIRT1, and its expression is critical for tumorigenesis. In fact, SIRT1 is overexpressed in chemoresistant leukemias, neuroblastomas and osteosarcomas, and in ovarian and breast cancer cells. Furthermore, ectopic SIRT1 overexpression confers cancer cells with resistance to the chemotherapeutic drug doxorubicin, whereas depletion of SIRT1 by siRNA partially reverses the drug-resistant phenotype (Chu et al. 2005). SIRT1 also sustains the cell proliferation signal and stimulates cell growth by regulating the MYC, p53 and FOXO pathways. Another important oncogenic role of SIRT1 is related to tumor promotion, through its role in angiogenesis: SIRT1 regulates vascular endothelial homeostasis by deacetylating FOXO1, Notch1 and endothelial nitric oxide synthase (eNOS). Furthermore, SIRT1 is activated in mammary epithelial cells during epithelial-to-mesenchymal transition (ETM)-like transformation and is a positive regulator of ETM and metastasis in prostate cancer. Finally, SIRT1 seems to regulate cancer-cell metabolism by stabilizing HIF-1α and HIF-2α, thereby promoting cell growth and proliferation (Roth and Chen 2014).

Similarly to SIRT1, SIRT2 might act as either tumor suppressor or tumor promoter. SIRT2 is reduced in gliomas, esophageal adenocarcinomas, gastric adenocarcinomas, and head and neck squamous cell carcinomas. Contrarily, its reduction is associated with increased apoptosis in gliomas and HeLa cells, and it is upregulated in several MYC-induced malignancies (Yuan et al. 2013). Two mouse models support a role for SIRT2 as a tumor suppressor: the first model develops spontaneous gender-specific tumors (e.g. mammary tumors in females; and liver, lung, pancreas, stomach, duodenum or prostate tumors in males) (Kim et al. 2011); the second model does not spontaneously develop cancer but is more prone to tumorigenesis under genotoxic conditions (Serrano et al. 2013).

SIRT3 is critical in metabolic homeostasis and in oxidative stress response, and is a mitochondrial tumor suppressor. However, although its protein levels are reduced in numerous cancers, some evidence suggests that it might actually function as tumor promoter. For example, SIRT3 levels are increased in lymph-node-positive breast cancer and in oral squamous cell carcinomas (OSCCs). SIRT3 inhibition sensitizes OSCC cells to radiation and to cisplatin treatment *in vitro*. As previously mentioned, SIRT3 protects cells from oxidative stress by various mechanisms, including activation of IDH2. However, activation of IDH2 in cancer might have a pro-survival effect (Yuan et al. 2013). Albeit $Sirt3^{-/-}$ mice initially appear normal (Lombard et al. 2007), they ultimately develop estrogen- and progesterone-positive mammary invasive tumors resulting from increased oxidative damage during aging (Kim et al. 2010). Accordingly, in $Sirt3^{-/-}$ mice the protection that CR affords against oxidative stress is diminished (Qiu et al. 2010).

All evidence points to SIRT4 being a tumor suppressor. SIRT4 levels are reduced in numerous human cancers, including breast, colon, gastric, ovarian and thyroid carcinomas (Yuan et al. 2013). Furthermore, $Sirt4^{-/-}$ mice develop lung tumors, which can be explained by the importance of SIRT4 in DNA damage response (Jeong et al. 2013). In cancer cells, mammalian target of rapamycin complex 1 (mTORC1) promotes degradation of SIRT4, thereby impairing its negative

function in glutamine anaplerosis. Thus, cancer cells exhibit increased glutamine metabolism and consequently, proliferate more rapidly (Csibi et al. 2013).

As mentioned above, Sirt6$^{-/-}$ mice exhibit an aging-like phenotype characterized by genome instability and metabolic defects. A growing body of research supports a role for SIRT6 as tumor suppressor, through its ability to control cancer metabolism, DNA repair and cell survival. SIRT6 is downregulated in several human cancers such as hepatocellular carcinoma, acute myeloid leukemia, head and neck squamous cell carcinoma, pancreatic cancer, and colorectal cancer. Consistently, one of the main SIRT6 targets, H3K56Ac, is hyperacetylated in breast, liver, skin, thyroid and colon cancers. Moreover, SIRT6 expression levels correlate with cancer progression and prognosis. In one study on colorectal cancer, patients with low SIRT6 levels were more likely to relapse and to present a shorter time to relapse than were those patients with high levels of SIRT6. However, the most important case of SIRT6 functioning as tumor suppressor probably stems from its functional relationship with HIF-1α, through which it inhibits the switch to aerobic glycolysis and consequently, prevents the Warburg Effect. In mice, loss of SIRT6 leads to tumor formation, independently of activation of known oncogenes, whereas transformed Sirt6$^{-/-}$ cells display increased glycolysis and can form tumors when injected into severe combined immune deficient (SCID) mice. These data suggest that SIRT6 contributes to both establishment and maintenance of cancer (Sebastian et al. 2012).

Despite the aforementioned findings, some evidence corroborates SIRT6 being a tumor promoter. For instance, chronic lymphocytic leukemia patients exhibit high levels of SIRT6 expression, which has been associated with poor prognosis (Wang et al. 2011). Additionally, SIRT6 expression is apparently upregulated in squamous cell carcinoma, through mis-regulation of miR-34 (Lai et al. 2013). SIRT6 protein levels are also elevated in paclitaxel- and epirubicin-resistant MCF-7 cells, and SIRT6 depletion sensitizes these cells to both drugs. Consistently, high SIRT6 protein levels in breast cancer patient samples have been significantly associated with poorer overall survival (Khongkow et al. 2013). Altogether, these findings suggest that in certain tumors, SIRT6 might play an oncogenic role that supersedes its metabolic role.

Treatment of specific cancers based on the metabolic role of SIRT6 might be a valuable therapeutic strategy. Interestingly, SIRT6 overexpression induces massive apoptosis in various cancer cell lines but not in normal, non-transformed cells. This apoptosis is mediated by the activation of both the p53 and the p73 apoptosis pathways, via the mono[ADP-ribosyl]transferase activity of SIRT6 (Fig. 5.2b). These results suggest that SIRT6 might be a promising target for cancer therapy (Van Meter et al. 2011).

Sirt7$^{-/-}$ mice present an aging-like phenotype and increased sensitivity to oxidative stress, which suggests a link to genomic protection. In contrast, SIRT7 is upregulated in various human cancers (e.g. breast, thyroid and liver cancers). Another important link between SIRT7 and cancer is the specificity of this Sirtuin for H3K18Ac. Global hypoacetylation of H3K18 has been linked to the ability of the adenovirus small early region-1a (E1A) protein to trigger oncogenic transformation (Ferrari et al. 2008; Horwitz et al. 2008). Moreover, hypoacetylation of

H3K18 is found in different cancers and has been linked to poor prognosis (Seligson et al. 2005). Thus, knockdown of Sirt7 in cancer cells reduces cell growth by inhibiting deacetylation of H3K18 (Barber et al. 2012). In contrast, SIRT7 promotes rRNA expression and therefore, favors proliferation. Thus, SIRT7 depletion leads to reduced rRNA synthesis, which in turn has been associated to decreased viability and proliferation in cancer cells. Interestingly, SIRT7 apparently maintains the phenotype of cancer cells at different levels, but its overexpression does not cause oncogenic transformation of immortalized mouse or human fibroblasts.

All of the aforementioned evidence supports an important role for Sirtuins in cancer initiation and progression. Given their involvement in tumor progression, Sirtuins are not only very promising targets for cancer therapy, but they also have potential as tumor biomarkers (depending on tumor type and stage). Overall, the evidence supports a promising future for Sirtuins in biomedicine, not only in the treatment of cancer but also in therapeutic strategies for other areas of human health.

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Chapter 6
MicroRNA Regulated Stress Responses in Cancer

Haoran Li, Shaan Gupta, and Burton B. Yang

Abstract Cancer cells often face unique challenges as they attempt to thrive in the human body, as a result of internal or external stressors. They are often faced with two options—adapt or perish. Their responses are usually the manifestation of complex molecular signaling cascades, which are attempting to maintain cellular homeostasis despite the increasingly harsh environment. These signaling cascades are fine-tuned through constant monitoring and regulation of genes, transcripts and proteins involved. As research elucidates the participants in these complex networks, microRNAs are emerging as key players in the regulation of stress responses in cancer highlighting a potential for the exploitation of these oligonucleotides for therapeutic use. There are thousands of microRNAs, each regulating hundreds to thousands of protein's expression levels, and this review serves to elucidate the nature of microRNAs through selected examples suggesting potential therapeutic opportunities.

Keywords microRNA • Cancer • Tumour • Stress response • Oxidative stress • Metabolic stress • Autophagy • Immune response • Cancer stem cells • Chemotherapy • microRNA-based therapies • Radiation therapy • Drug resistance

6.1 Introduction

MicroRNAs (miRNAs) are single stranded, short sequence, non-coding RNAs that are broadly conserved across species. So far, more than 1,400 miRNAs have been identified in the human genome (Kozomara and Griffiths-Jones 2011). Most miRNA loci are found within the introns of protein coding genes, while they can be also embedded in exonic regions or separate transcriptional units (Jansson and Lund 2012). In most cases, miRNA genes are transcribed by RNA polymerase II (pol II)

H. Li • S. Gupta • B.B. Yang (✉)
Sunnybrook Research Institute, Sunnybrook Health Sciences Centre,
S110, S-Wing Research Building, 2075 Bayview Ave, Toronto M4N 3M5, ON, Canada

Department of Laboratory Medicine and Pathobiology, University of Toronto,
Toronto, ON, Canada
e-mail: byang@sri.utoronto.ca

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in the nucleus, where they are then processed by the complex containing RNase III enzyme Drosha and co-factor Di George syndrome critical region 8 (DGCR8). Drosha-DGCR8 complex trims the long primary miRNAs (Pri-miRNAs) into 70–100 nt long precursor miRNAs (Pre-miRNAs), which are subsequently exported out of the nucleus by exportin-5 (XPO5). In the cytoplasm, pre-miRNAs hairpins are cleaved by a protein complex including RNase III-type enzyme Dicer and the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), giving rise to double-stranded RNAs (ds-RNAs) approximately 22 nt long. This dsRNAs include two miRNA strands, known as miRNA-3p and miRNA-5p, in both arms of the pre-miRNAs. It was used to think that one strand is a mature miRNA and the other strand (the passenger strand) is normally subject to degradation, yet the current evidence suggests that either arm can be selected as a mature miRNA in a tissue-specific context (Shan et al. 2009; Kahai et al. 2009). The mature single-stranded miRNAs are incorporated into the RNA induced silencing complex (RISC), which contains core components such as argonaute 2 (AGO2), Dicer and TRBP (MacRae et al. 2008). RISC is responsible for inducing posttranscriptional gene silencing by base-pairing to partially complementary sequence motifs within 3′ untranslated regions (3′-UTR) of target mRNAs. As such, they are able to cleave the mRNAs directly, enhancing mRNA degradation, or to repress mRNA translation. Some studies also showed that RISC activated mRNA translation by binding to the 5′-UTR of target mRNA (Vasudevan et al. 2007). More recently, some miRNAs have been found to bind decoy mRNAs in a RISC-independent way (Lee et al. 2007). The decoy transcripts in turn regulate miRNA activities (Lee et al. 2010, 2011; Rutnam and Yang 2012a; Rutnam et al. 2014).

The first miRNA, lin-4, was identified in 1993 by Victor Ambros and colleagues in a study of C. elegans development (Lee et al. 1993) (Fig. 6.1). However, it was not until 1998 that the mechanism of RNA interference was unprecedentedly illuminated by Craig C. Mello and Andrew Fire (Fire et al. 1998). In this work, they

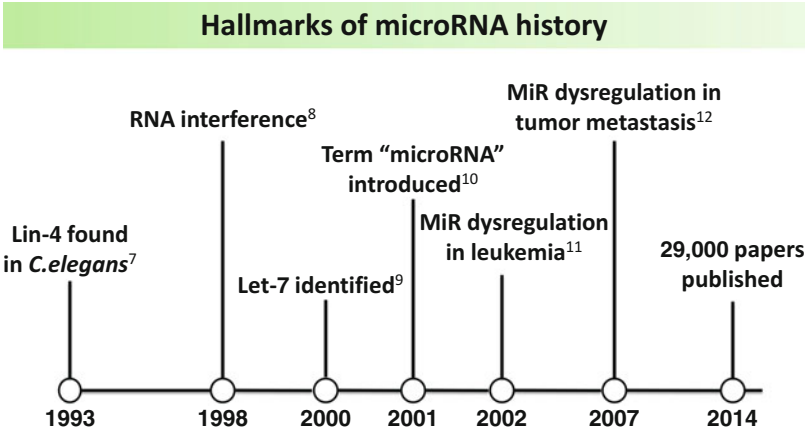

Fig. 6.1 A brief history of microRNA research milestone

found double-stranded RNA was surprisingly more effective at producing interference than either single-stranded mRNA or antisense RNA, and thereby they named this phenomenon as RNAi. This study is an important contribution to understanding how the miRNA-RISC complex functions to inhibit gene expression. Soon after, RNA interference (RNAi) pathways were found to play critical roles in development, cell proliferation, differentiation, and stress response. In 2000, the second small regulatory RNA let-7 was identified as a developmental regulator, sparking an explosive research interest focusing on C. elegans, plants and animals (Reinhart et al. 2000). Several more small expressed RNAs were found in 2001 and then coined the term "miRNA" (Lagos-Quintana et al. 2001). The roles of miRNAs in the development of human cancer was not established until 2002, when Croce and colleagues found both miR-15 and miR-16 are located in chromosome 13q14, and they are down-regulated in approximately 68 % chronic lymphocytic leukemia patients (Calin et al. 2002). Over the last decade, many miRNAs have been implicated in human cancer development (Rutnam and Yang 2012b; Rutnam et al. 2013; Yang et al. 2011a). Interestingly, their genes are located mostly near cancer susceptibility loci. Mapping of miRNA genes provides specific clue for the possible roles of miRNA in tumorigenesis events (Croce 2009).

The function of miRNAs requires a sequence specific match to their target mRNA. The majority of match pairs are composed of 7–8 nt nucleotides in the "seed" regions (5′-end) of the miRNAs that are perfectly complementary to 3′-UTR segments of the target mRNAs (Bartel 2009). The mechanistic model of "seed" pairing leads to the possibility that miRNAs are influencing the expression or evolution of nearly all mammalian mRNAs (Farh et al. 2005). It is well established that miRNAs are broadly involved in cancer cell proliferation, tumorigenesis, metastasis, angiogenesis and drug resistance. Based on the influence on cancer cell growth, they can be categorized as oncogenic or tumor-suppressive miRNAs. Oncogenic miRNAs (oncomirs) induce cancer cell proliferation by down-regulating expression of tumor suppressor genes, whereas tumor suppressor miRNAs (mirsupps) inhibit cancer progression by targeting oncogenes post-transcriptionally (Li and Yang 2013a). However, this dichotomous approach is challenged by growing evidence. A particular miRNA could be increased in some cancers as an oncomir, but downregulated in other cancers. For example, miR-17 was found as a mirsupps in breast cancer (Hossain et al. 2006). while it promotes development of hepatocellular carcinoma (Shan et al. 2013). Moreover, as a single miRNA is able to target a host of mRNAs, studying miRNA's function is complicated by enormous genetic diversity observed in cancers. Hence, miRNAs and their related network more likely have a buffering effect in cellular hemostasis.

6.2 MicroRNA and Metabolic Stress in Cancer

The growth of cancer requires increased supply of nutrition and oxygen, which permits rapid expansion of a tumor. To adapt to the accelerated metabolism rate, cancer cells develop unique genetic alternations that dysregulate the control of cell

proliferation. Perhaps one of the significant adaptations is Warburg effect, which is named after Dr. Otto Warburg. His discovery that cancer cells harbor a highly glycolytic rate which increases glucose consumption and lactate production regardless of the concentrations of oxygen, giving rise to a new era where detection and treatment of cancer could be focused on its unique metabolic signature (Warburg 1956). Thus some researchers suggest that cancer as a whole is a metabolic disease.

6.2.1 MicroRNA and Oxidative Stress

As a result of Warburg effect and anaerobic respiration, several potential toxic compounds are generated. They include reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS) and reactive chloride species (RCS) (Sosa et al. 2013). Of them, ROS are produced most abundantly. These reactive species can cause damage to DNA structure and its repair mechanism. They can also liberate lipid peroxidation and increase permeability of cell membrane. Elevated concentration of ROS has been frequently found in cancer cells. Oxidative stress affects several biochemical pathways, such as PTEN/PI3K/Akt, and MAPK/ERK. Notably, miRNAs also actively respond to intracellular change of ROS. It was firstly identified that ROS accumulation in small cell lung cancer (SCLC) cells was linked with miR-17-92 (Ebi et al. 2009). Overexpression of miR-17-92 cluster counterbalances ROS generation in SCLC cells. It was suggested that miR-17-92 plays a role in fine-tuning the effects of ROS-induced DNA damage, maintaining genomic stability (Ebi et al. 2009). Several ROS-related miRNAs have been described thereafter (Sosa et al. 2013). MiR-200 family—comprising miR-141, miR-200a, miR-200b, miR-200c and miR-429—has been shown as key regulators of oxidative stress (Batista et al. 2013) (Fig. 6.2). These miRNAs control cellular motility by mediating epithelio-mesenchymal transition (EMT), and they also influence cellular stemness and apoptosis by targeting p38α MAPK. High expression of miR-200 s is often found in epithelial ovarian cancer (EOC) and correlated with a better outcome and/or early-stage disease (Marchini et al. 2011). Based on the level of miR-200, EOC can be stratified as "oxidative stress" and "fibrosis" signature (Batista et al. 2013) "Stress" patients have a better response to chemotherapy and longer survival, compared to "fibrosis" patients. There was an enhanced expression of miR-141 and miR-200a in ovarian cancer cells exposed to oxidative stress, leading to down-regulation of p38α and increased ROS production. The up-regulation of ROS levels, in turn, augments expression of miR-200 family, which together sensitizes tumor cells to cisplatin or carboplatin treatment (Mateescu et al. 2011). This study implicates that the signature of miR-200s can be used as predictive biomarker for chemotherapy response. Restoration of miR-200s levels may be a new therapeutic approach in drug resistant EOC patients.

Increased levels of glycolysis and anaerobic respiration prevent tumor cells from entering senescence and stimulate vascularization. Accumulating studies have

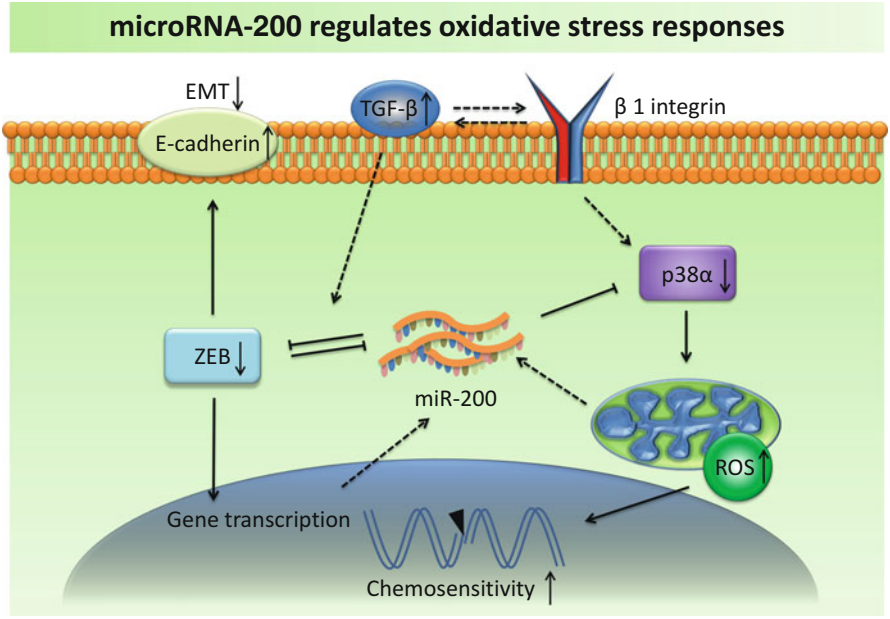

Fig. 6.2 Increased expression of miR-200 suppresses ROS-inhibitor p38α and EMT-inducer ZEB, which in turn regulates miR-200 in a feedback loop

demonstrated the role of miRNAs in regulating cellular response to hypoxia. Most of these hypoxia-responsive miRNAs are found to be associated with hypoxia-inducible factor 1 (HIF-1) signaling pathway. MiR-210 is a robust target of HIF-1, and its overexpression has been linked to adverse prognosis in breast cancer and hepatocellular carcinomas (Devlin et al. 2011). It is indicated that miR-210 activates the generation of ROS, by targeting ISCU (iron-sulfur cluster scaffold homolog) and COX10 (cytochrome c oxidase assembly protein), two key factors of the mitochondrial electron transport chain. It thus inhibits mitochondrial function and up-regulates the levels of glycolysis (Chen et al. 2010). ISCU, which is also a target of HIF-1, is a cofactor for enzymes involved in the TCA cycle and iron metabolism. Through interfering with HIF-1 at multiple levels, miR-210 enhances cancer cell survival in hypoxic condition, but also makes cells more sensitive to glycolysis inhibitor (Ying et al. 2011).

6.2.2 MicroRNA and Starvation

It is well known that tumor cells are characterized by high nutritional requirements underlying a constitutively 'hungry' phenotype. In response to nutrient starvation, varied changes will occur on the genetic and epigenetic levels favoring cell survival. It was firstly found that miRNA was involved in starvation-induced alternations in

human hepatocarcinoma cells (Bhattacharyya et al. 2006). When cells are growing under unstressed conditions, miR-122 binds to the 3′-UTR of cationic amino acid transporter 1 (CAT-1) mRNA. Nevertheless, such pairing was reversed in cells subject to starvation, by relocalization of CAT-1 mRNA from cytoplasmic processing bodies (PBs). As scaffolding center of miRNA function, the activity of PBs showed an on- and off- switch in a context-dependent manner. Under stressed conditions such as amino acid deprivation, the 3′-UTR of CAT-1 binds to HuR, an ARE binding protein, relieving CAT-1 from miR-122 suppression in PBs and recruiting it to polysomes for translation (Bhattacharyya et al. 2006). This model suggests a way that RNA-binding protein modulates the activity of miRNAs in tumor cells under stressed conditions. Through promoter region-directed modulation, miRNA activation is closely related to the intracellular environment. It is found that glucose depletion-induced oxidative stress inhibits histone deacetylation in miR-466h-5p promoter region, which actives miR-466h-5p, miR-669c and Sfmbt2 in a time-dependent manner (Druz et al. 2012). The authors suggested that miR-297-669 cluster, including miR-466h-5p, might play a role in cellular detoxification and drug-induced injuries. During oncogenic transformation, induction of miR-297-669 cluster is inhibited with the loss of oxidative stress defense mechanism (Druz et al. 2012).

Glioblastoma cells are characterized by an aggressive growth pattern and frequent cellular apoptosis, making it an optimal model to test the nutrition-dependent functions of microRNAs. In cultured U87 cells deprived of serum, the levels of miR-17 increased remarkably (Fig. 6.3). By targeting PTEN and stabilizing HIF-1

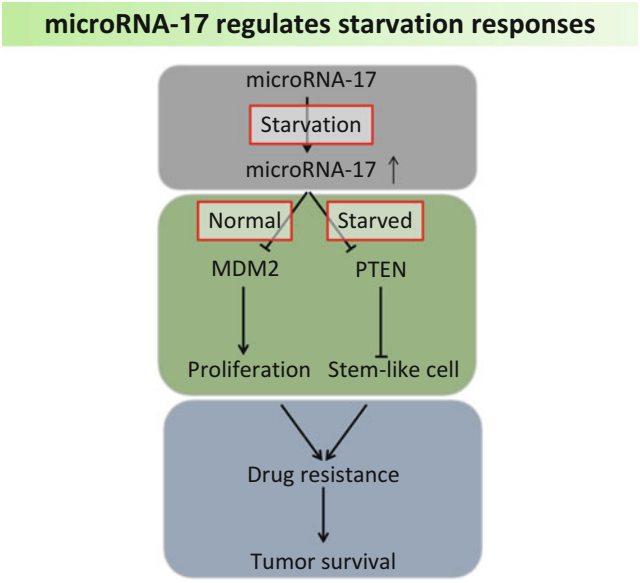

Fig. 6.3 MicroRNA-17 expression increases under starved conditions, which further facilitates tumor survival by targeting MDM2 and PTEN

alpha, miR-17 reduced cellular metabolic rates under unfavorable conditions in order to protect them from starvation. Notably, miR-17 also inhibited tumor cell proliferation under unstressed conditions through targeting MDM2, an oncogene often over-expressed in cancer cells. Thus, miR-17-overexpressing cells became more resistant to chemotherapy, since most cytotoxic reagents act by diminishing highly proliferative cells (Li and Yang 2012). This finding, in which miR-17 plays a dual role in glioblastoma cells, provides a new perspective to our understanding of stress responses in cancer. MiR-93, a paralog of miR-17-92, was found increased in human breast carcinoma. By modulating large tumor suppressor homology 2 (LATS2), miR-93 enhances tumor angiogenesis and metastasis in a mouse lung metastasis model (Fang et al. 2012). It also promotes tumorigenesis and angiogenesis in human brain tumor by targeting integrin beta-8 (Fang et al. 2011). These findings suggest a promising role of miRNA as a predictor in "tumor-starving" therapy. Since tumors harboring highly expression of these miRNAs often show an excessively angiogenesis pattern, elucidation of the underlying cross-reaction between miRNAs and anti-angiogenic treatment is likely uncover new opportunities for therapeutic intervention.

In order to survive under conditions of high nutrient demands ('hungry state'), cancer cells heavily rely on aggressive angiogenesis to permit ample blood supply and oxygen uptake. Therefore, "tumor-starving" (anti-angiogenic) therapy has been employed to prevent tumor vascularization and deprive it from nutrition. Initially, it was found that miR-378 contributes to tumor angiogenesis in transplanted glioblastoma, by targeting SuFu and Fus-1. As a result, miR-378 promotes tumor cell survival and growth (Lee et al. 2007). Furthermore, miRNA-induced angiogenesis is a common phenomenon observed in different types of tumor (Li and Yang 2013b).

6.2.3 MicroRNA and Autophagy

Autophagy is a catabolic process which transports cellular components to lysosomes for self-degradation. It is a cytoprotective mechanism in maintaining hemostasis and highly conserved during evolution. Deregulation of autophagy has been implicated in a variety of cancers (Xu et al. 2012). Due to elevated metabolic demands, aggressive tumor cells often display robustly activated autophagy in order to fuel mitochondrial metabolism. Autophagy may also limit ROS toxicity by triggering mitophagy, wherein damaged mitochondria are eliminated (Mathew and White 2011). Beclin 1 (also known as autophagy-related gene 6 or Atg 6) is a key autophagy-promoting player in development and progression of cancer, including breast cancer, ovarian cancer, glioblastoma and lymphoma (Huang et al. 2010). It was firstly demonstrated that Beclin 1 is a potential target of miR-30a. Inhibition of beclin 1 expression by miR-30a blunted the activation of autophagy induced by rapamycin in tumor cells (Zhu et al. 2009). The miR-30 cluster contains five paralogs: miR-30a, b, c, d, e. The potential targets of miR-30 subfamily also include

B-Myb, a transcription factor that positively regulates cell proliferation and cell cycle. By binding to the 3′-UTR of B-Myb, miR-30 is able to repress endogenous expression of B-Myb and inhibit cellular senescence in Hela cells (Martinez et al. 2011). Further investigation suggests that miR-30 is a prominent tumor suppressor in prostate cancer, breast cancer and glioblastoma (Kao et al. 2014). Through regulation of EMT-associated oncogenes, miR-30 in prostate cancer cells suppresses EMT features and inhibits tumor cell migration and invasion (Kao et al. 2014). Remarkably, miR-30 is also broadly involved in tumor cell apoptosis and stem-like cells generation (Yu et al. 2010). Taken together, these data suggest that miR-30 represents a bridge between apoptosis and autophagy (Xu et al. 2012).

Another well-studied miRNA is the miR-17-92 cluster. The human miR-17-92 cluster locates at 13q31.3, a fragile region often amplified in hematopoietic malignancies. The function of this cluster was firstly reported in B-cell lymphoma, where enforced expression of miR-17-92 accelerated tumor development by acting with c-myc (He et al. 2005). Cumulative evidence has demonstrated a pivotal role of miR-17-92 in cancer. As a potential oncomir, miR-17-92 was found abundantly expressed in immature hematopoietic cells. Sequestosome 1 (SQSTM1), an ubiquitin-binding protein associated with autophagy, was found inhibited by miR-17-92 cluster in myeloid progenitors (Meenhuis et al. 2011). SQSTM1 plays an important role in inclusion body formation by binding to the autophagic regulator Atg8/LC3 (Komatsu et al. 2007). In tumor cells under stress, accumulation of SQSTM1 caused persistent damage to mitochondria and cellular genome. It was indicated this failure to eliminate SQSTM1 was sufficient to alter NF-kB pathway and contribute to tumorigenesis (Mathew et al. 2009). By interfering with SQSTM1-regulated pathways, miR-17-92 actively modulates stress responses in tumor cells.

In addition, miR-155 also was found to play a role in autophagy by regulating multiple molecules including RHEB, RICTOR, and RPS6KB2 in the mTOR signaling pathway (Wan et al. 2014). Increased expression of mIR-155 increases autophagic activity in human nasopharyngeal cancer and cervical cancer cells. Silencing endogenous mIR-155 inhibits hypoxia-induced autophagy. These results release a new role for mIR-155 as a key regulator of autophagy via dysregulation of MTOR pathway.

On the other hand, components involved in the miRNA biogenesis pathway are closely linked with autophagy process (Fullgrabe et al. 2014). As a functional center of miRNA-RISC complex, DICER1 and AGO2 can be integrated in the autophagosome after binding to the selective autophagy receptor NDP52 and GEMIN4 (Fullgrabe et al. 2014). It is eventually leading to protein degradation in the autophagosome-dependent lysosome. Therefore, autophagy is involved in maintaining miRNA biogenesis by removing inactive DICER1-AGO2 complex, preventing them competing for additional factors which are required for miRNA maturation (Gibbings et al. 2012). In turn, miRNAs control the activity of core autophagy proteins. In chronic lymphocytic leukemia cells, miR-130a inhibits autophagosome generation by targeting DICER1 and ATG2B (Kovaleva et al. 2012). It was indicated that miR-130a and DICER1 form a regulatory feedback loop that mediates tumor cell survival (Kovaleva et al. 2012).

6.3 MicroRNA and Tumor Microenvironment

Tumor microenvironment comprises blood vessels, immune cells, fibroblasts and extracellular matrix. Numerous signaling molecules and pathways are influencing the interactions between the tumor and its surrounding microenvironment. It is believed that such interplay is remolding tumor microenvironment, which allows for tumor angiogenesis and metastasis. Meanwhile, immune responses are often suppressed in the host, leading to tumor-tolerogenic macrophages, NK/T cells and neutrophils. Any fluctuation in microenvironment could impact global signaling of tumor cells, and thus influence the stress response through miRNA-regulated pathways.

6.3.1 MicroRNA and Immune Response

The puzzling question how tumor cells escape from natural immune surveillance has stimulated extensive research into tumor mediated immune suppression. It is becoming increasingly clear that immune response dysregulation plays a critical role in cancer progression and therapeutic resistance. Hence normalizing of the microenvironment can improve anticancer outcome. Analysis of tumor infiltrating lymphocytes has demonstrated that many types of tumors show evidence of T-cell infiltration (Gajewski et al. 2013). Of particular, activated CD8+ T cell responses have been associated with a positive prognosis in tumors such as colorectal cancer (Mlecnik et al. 2011). More studies are underway to explore the prognostic value of cancer associated immune biomarkers. Recent findings have suggested that miRNAs are greatly involved in modulating the proliferation, differentiation and response of CD8+ T cells. Initial characterization of miRNA profile in CD8+ T cells provided insight into the understanding of miRNA's role in a cell-specific setting (Fig. 6.4).

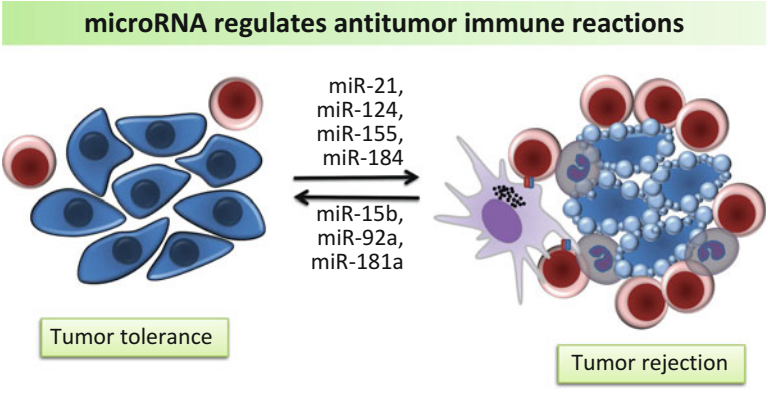

Fig. 6.4 MicroRNAs are involved in regulation of tumor tolerance and antitumor immune reaction by mediating CD8+ T cells, NK cells and macrophages

Comparing naïve, effector, and memory CD8+ T cells, it was shown that 7 miRNAs (miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-7f) are most frequently expressed in all the T cell subsets, whereas they tend to be down-regulated in effector T cells and come back up in memory T cells (Wu et al. 2007). During the process of differentiation, some miRNAs such as miR-21 and miR-155 are found up-regulated while the miR-17-92 cluster is concomitantly decreased (Salaun et al. 2011). T cell tolerance to cancer cells characterizes immune suppression in the tumor microenvironment. Rescuing tolerant T cells by lymphopenia-mediated homeostasis-driven proliferation may enable development of new immunotherapeutic strategies. By analyzing genome-wide miRNA profile in tolerant T cells, Greenberg et al. found that miR-21 and miR-184 up-regulated after rescuing, whereas miR-181a was decreased (Schietinger et al. 2012). Further studies revealed that miR-181a expression inversely correlated with mRNA levels of 56 predicted target genes. The authors pointed out that miR-181a could be a possible key negative regulator of functions in CD8+ T cells (Schietinger et al. 2012). By inhibiting innate immune response, miR-181 may enhance tumor vascular invasion and metastasis. Over-expression of miR-181 was found correlated with poor survival in oral squamous cell carcinoma, suggesting it as a potential biomarker for cancer tolerance and prognosis (Yang et al. 2011b). The understanding of miRNA's potent effects in tumor-mediated immunosuppression was driven by studies in tumor-bearing mice. Increased expression of miR-15b was observed in isolated CD8+ T lymphocytes in mice with Lewis lung carcinoma (Zhong et al. 2013). Ectopic expression of miR-15b in CD8+ T cells inhibits apoptosis by knocking down death effector domain-containing DNA-binding protein (DEDD). DEDD is a ubiquitous death effector domain containing protein which induces apoptosis through its N-terminal DED motif. High expression of miR-15b is also associated with inactivation of CD8+ T lymphocytes by repressing the production of cytokines such as IL-2 and IFN-γ (Zhong et al. 2013). Despite of its anti-apoptotic effect, miR-15b likely plays a negative role in the activation of effector T cells and anti-tumor immune response. Dynamic change of tumor-associated miRNA expression can be also observed in miR-17-92 cluster. In patients with multiple myeloma, the miR-92a level in CD8+ T cells was significantly down-regulated compared with normal subjects (Yoshizawa et al. 2012). With the remission of disease, the plasma miR-92a levels became normalized. Together, these findings suggest gain or loss of miRNA functions may represent the T-cell immunity status in tumor host.

Accumulating evidence has identified signal transducer and activator of transcription 3 (STAT3) as a critical molecule in regulating tumor-associated immunosuppression by interfering with multiple factors. Constitutive expression of STAT3 alters gene-expression programs, inhibits expression of immune mediators and suppresses leukocyte infiltration into the tumor (Yu et al. 2007). Blocking STAT3 in immune cells can generate diverse anti-tumor immunity by suppressing negative regulators such as immature dendritic cells and regulatory T cells and activating CD8+ T cells, natural killer cells and neutrophils (Yu et al. 2007). Thus, STAT3 has emerged as a potential target for tumor immunotherapy. Recent studies have

demonstrated that the interplay between miRNAs and STAT3 broadly exists in cancer development and progression. MiR-124 has been reported as a potential tumor suppressor in diverse tumor types, such as colorectal cancer and prostate cancer (Cao et al. 2013). In patients with glioblastoma, miR-124 expression is significantly reduced, compared to normal brain tissues (Wei et al. 2013). Ectopic upregulation of miR-124 in glioma stem-like cells promoted T cell proliferation and regulatory T cell induction. Moreover, treatment of T cells from glioblastoma patients with miR-124 induced pro-inflammatory cytokines and chemokines (Wei et al. 2013). As a result, systemic administration of miR-124 prolonged overall survival and decreased tumor incidence in a murine glioma model. Such anti-tumor effects were proved to depend on the presence of T cells. In tumor bearing mice depleted of CD4+ or CD8+ cells, the immunotherapeutic effects of miR-124 was ablated (Wei et al. 2013). Activation of STAT3, in turn, can modulate expression of several miRNAs. For example, there is a highly conserved STAT3-binding site in the promoter of the miR-17-92 gene (C13orf25) (Brock et al. 2009). By modulating the expression of IL-6, activation of STAT3 upregulates the entire miR-17-92 cluster. Interestingly, there are two seed regions of miR-17-92 in STAT3 3′-UTR, and thereby miR-17-92 reversely targets STAT3 expression, leading to reduced ROS generation (Zhang et al. 2011). By modulating STAT3 associated immune tolerance in myeloid-derived suppressor cells (MDSCs), the negative regulatory loop between miR-17-92 and STAT3 may be an important factor in tumor associated immune response and a potential immunotherapeutic target against cancer.

6.3.2 MicroRNA and Epithelial Mesenchymal Transition

Epithelial mesenchymal transition (EMT) is regarded as a key process of tumor invasion and distant metastasis. It is essential for cancer cells facilitating survival in a hostile milieu and escape from adverse sites (Taddei et al. 2013). As a major stress-adaptive strategy, EMT leads epithelial cells to lose their cell polarity and cell-cell adhesion, and gain morphological and functional characteristics of mesenchymal cells (Taddei et al. 2013). A prominent marker of EMT is the loss of epithelial cadherin (E-cadherin) expression. E-cadherin is a transmembrane glycoprotein that mediates intercellular adhesion via hemophilic binding (Sheng et al. 2006). Inactivation of E-cadherin has been found in most carcinomas (Paredes et al. 2012). Recently, miRNAs are emerging as potential regulators of E-cadherin. MiR-200 family is the first miRNAs identified to be associated with E-cadherin expression (Hurteau et al. 2007). In breast cancer cells with less invasive phenotype, there is endogenous expression of both miR-200c and E-cadherin. However, in estrogen receptor negative cells, miR-200c as well as E-cadherin levels are merely detectable. By targeting E-cadherin repressors ZEB1 and ZEB2, miR-200c was able to restore E-cadherin function and therefore inhibit EMT (Hurteau et al. 2007). Most recent study revealed that miR-200/ZEB interaction is

crucial to breast cancer growth and metastasis (Truong et al. 2014). This network is subject to the regulation of β1 integrin and transforming growth factor-β (TGF-β). In triple negative breast cancer cells, knockdown of β1 integrin changed cell migration pattern and stimulated distant metastasis by activating TGF-β. Reducing the abundance of TGF-β or restoring the ZEB/miR-200 balance reestablished cell cohesion and reduced tumor dissemination (Truong et al. 2014). Augment evidence suggests miR-200 as a potential marker of metastasis capacity. MiR-200/ZEB network is not only involved EMT in breast cancer, but also a key regulator of prostate cancer and gastric cancer (Williams et al. 2013). Imbalance of miR-200/ZEB is associated to invasive subtype of gastric cancer and poor prognosis of patients (Song et al. 2014). Notably, miR-200 family also exerts their effects on cellular plasticity and metastasis by modulating additional signaling in parallel to ZEB. Actin-associated gene moesin was inversely associated with miR-200 expression (Li et al. 2014). In a similar pattern as miR-200/E-cadherin interaction, miR-200/moesin axis regulates breast cancer cell metastasis in a context-dependent manner (Li et al. 2014).

6.4 MicroRNA Regulation of Stress Responses to External Stimuli in Cancer

When a patient is first diagnosed with cancer, the common medical practice is to perform surgical resection of the tumour, in an attempt to liberate the patient from the cancerous mass of cells. Unfortunately, tumour resection is not always an optimal procedure for cancer on its own, and despite the surgeon's best efforts, cancer cells will still be present in the patient's body, and will continue to grow if left untreated. In an attempt to combat these remaining cells, chemotherapy or radiotherapy is often used adjunctively. Each of these procedures induces a molecular stress response, as the patient's remaining tumour cells attempt to survive the poisons and high-energy radiation that they are being bombarded with. The cells are faced with two options—adapt or perish. These responses are usually the manifestation of complex molecular signaling cascades, which are attempting to maintain cellular homeostasis despite the increasingly harsh environment. These signaling cascades are fine-tuned through constant monitoring and regulation of the genes, transcripts and proteins involved. As research elucidates the participants in these complex networks, microRNAs are emerging as key players in the regulation of stress responses to chemotherapy and radiotherapy highlighting a potential for the exploitation of these oligonucleotides for therapeutic use. There are thousands of microRNAs, each regulating the levels of hundreds to thousands of proteins, and this review aims at illuminating the nature of microRNAs through select examples, thus elucidating possible therapeutic opportunities.

6.4.1 MicroRNA and Chemotherapy

Chemotherapy is a cancer treatment generally involving the use of one or more cytotoxic drugs with the aim of slowing and ideally stopping the growth of tumours. Chemotherapeutics generally act by targeting rapidly dividing cells (which cancer cells are) and preventing cell division through a variety of mechanisms including impairing the cell division machinery and damaging DNA—often leading to programmed cell death, known as apoptosis.

Chemotherapy can offer an excellent adjuvant treatment for killing cancer cells. However, this treatment often becomes less effective, as cancer cells acquire traits helping them to survive the toxicity. Resistance to chemotherapy is believed to cause treatment failure in over 90 % of patients with metastatic cancer (Longley and Johnston 2005). Resistance to the stressors of chemotherapy can occur through many different mechanisms, which are poorly understood. However, it is becoming increasingly apparent that microRNAs can serve a regulatory role in the molecular mechanisms underlying drug resistance and thus may hold the potential to be used to reverse chemoresistance.

6.4.1.1 Regulation of Multidrug Resistant Proteins

The ATP-binding cassette (ABC) transporter is a superfamily of transmembrane proteins transporting compounds across the cellular membrane against the concentration gradient through the use of ATP-coupling. Nine members of the "C" subfamily of ABC transporters (entitled multidrug resistance proteins, or MRPs) have been found mediate chemotherapeutic drug resistance through efflux of the drug out of the cells (Chen and Tiwari 2011). By pumping the chemotherapeutic out of the cell via MRPs, cancer cells are able to minimize exposure to the toxic drugs.

Several studies have implicated microRNAs in the regulation of MRP levels, and thus more broadly implicate microRNAs in chemoresistance. For example, miR-27a was found to directly target and have the net effect of down-regulating the MRP p-glycoprotein expression and reversing chemoresistance in leukemic cells. Interestingly, a separate study found that miR-27a could activate p-glycoprotein indirectly (through the down-regulation of an upstream target) and contribute to chemoresistance in ovarian cancer cells. This seemingly contradictory data exemplifies the convoluted and often environment-specific effects of microRNA, of which a complete understanding is still in its infancy.

6.4.1.2 Regulation of Drug Metabolization

As chemotherapy drugs course through patients' vasculature, they are slowly metabolized by the liver, where they are rendered inactive and then eventually expelled from the body. The cytochrome P450 (CYP) superfamily is a group of

enzymes that catalyze the oxidation of organic molecules, and account for approximately 75 % of drug metabolism (Guengerich 2008).

Many microRNAs have been found to regulate the expression of CYPs, and thus regulate the rates of drug metabolization. As the rates of drug metabolization increase, the exposure of cancer cells to the chemotherapeutics decrease, thus decreasing overall cancer cytotoxicity and facilitating tumour survival. For example, miR-27b can target CYP1B1, and knocking down miR-27b has been shown lead to increased CYP1B1 protein expression (Tsuchiya et al. 2006). Increased levels of CYP1B1 have been implicated with resistance to the chemotherapeutic Docetaxel in cancer cells (Martinez et al. 2008). Moreover, studies using microRNA-based shRNA knockdown of CYP3A have demonstrated a dose-dependent inhibition of CYP translation (Wang et al. 2012).

6.4.1.3 Modulating the Cell Death Response

Chemotherapeutics often exert their ultimate effect on cancer cells by inducing intrinsic or extrinsic apoptosis. MicroRNAs are able to facilitate cancer cell survival by down-regulating proteins involved in the programmed cell death response, thereby facilitating the escape from effects of chemotherapy through yet another modality. For example, intrinsic/mitochondrial apoptosis is regulated by the B-cell lymphoma 2 (Bcl-2) family of anti-apoptotic proteins. MiR-125b has been shown to target and down-regulate Bcl-2 expression in breast cancer cells, thus conferring tumour suppression in hepatic cancer cells (Zhou et al. 2010). However, in breast cancer cells, miR-125b has been shown to target and down-regulate pro-apoptotic Bcl-2 antagonist killer (Bak1) expression, thus conferring these cells with chemoresistance to paclitaxel (Zhao et al. 2012). Once again, the complex environment-specific effect of microRNA is exemplified.

6.4.1.4 Influence on Targeted Therapies

Novel approaches to cancer therapy have shifted focus away from targeting all fast-dividing cells, and instead focus on blocking the growth of cancer cells by interfering with molecules specific to carcinogenesis and tumour growth. For example, in hormone receptor-positive breast cancer, the estrogen receptor is over-expressed, and estrogen is required for the cancer cells to grow. The targeted therapies tamoxifen and raloxifene act by blocking the estrogen receptor, thus blocking breast cancer cell growth. Interestingly, miR-206 and miR-221/222 have been shown to negatively regulate expression of estrogen receptor alpha, which has been associated with tamoxifen insensitivity in breast cancer cells (Adams et al. 2007; Zhao et al. 2008).

6.4.1.5 Regulation of Cancer Stemness

Cancer stem cells (CSCs) are cancer cells that are believed to possess stem-like properties, giving them the ability to develop into all cell types found in cancer samples. It has been proposed that most current chemotherapies fail to eradicate cancer cells altogether, largely because they fail to target the CSCs, which may evade anticancer drugs partly due to their slow-growing nature. Several microRNAs have been implicated in contributing to the stem-like properties of cancer stem cells. Glioblastoma cells exogenously over-expressing miR-378 were found to contain a larger group of side population cells with a high density of CSCs compared to the wild type control (Deng et al. 2013). The cells over-expressing miR-378 were found to grow slower, but had higher survival rates than the control when treated with chemotherapeutic agents (Li and Yang 2012).

6.4.2 MicroRNA and Radiotherapy

Radiation therapy is a cancer treatment that involves subjecting a patient's tumour to ionizing radiation to kill malignant cells. Ionizing radiation damages cells by producing intermediate ions and free radicals which cause double stranded breaks (DSBs). If left unfixed, this DNA damage leads to the death of the cells. Subjecting cells to ionizing radiation stimulates a stress response, whereby the cell undergoes a battery of molecular changes in attempts to mitigate the damage and repair damaged DNA (Metheetrairut and Slack 2013). Many of the molecular processes in this stress response have been shown to be regulated by microRNAs, which opens the possibility for them to be exploited as radio-sensitizers in the future.

6.4.2.1 Response to Damaging Radicals

As previously mentioned, the ionizing radiation produces free radicals, which exert their lethal effect on cancer cells by inducing double strand breaks, which may eventually lead to cell death. A radical is an atom that contains an unpaired valence electron, making the atom highly unstable and chemically reactive. These radicals can then attack the deoxyribose DNA backbone and cause DSBs. Thus, in attempt to mitigate these damaging effects, cells have developed mechanisms to metabolise harmful radicals. For example, the superoxide dismutase family of proteins catalyzes the degradation of the free radical superoxide anion to hydrogen peroxide. Studies have shown that miR-21 targets and down-regulates superoxide dismutase 3 protein expression. It also indirectly lowers superoxide dismutase 2 protein levels, and ultimately leads to higher levels of superoxide levels, which may act as a radio-sensitizer by permitting higher levels of DSBs (Metheetrairut and Slack 2013; Zhang et al. 2012).

6.4.2.2 Regulation of DNA Histone Modification

DNA is usually tightly coiled and packaged into a nucleosome, which can be thought of as a thread tightly wrapped around a spool. In order for the DNA repair machinery to physically access the DSB site, the DNA must first be unpackaged. H2AX is a member of the histone protein family, and phosphorylation of this protein leads to DNA that is less condensed to permit DSB repair (Fernandez-Capetillo et al. 2004). Both miR-24 and miR-138 have been shown to target H2AX, and overexpression of these microRNAs results in H2AX protein down-regulation, more DSBs and radiosensitivity (Lal et al. 2009; Wang et al. 2011).

6.4.2.3 Regulation of Cell Cycle

Cell cycle checkpoints are mechanisms that allow cells to ensure the integrity of their genome. In these highly regulated processes, DNA damage leads the cells to undergo cell cycle arrest, which allows the cells to repair the DSB. The cyclin-dependent kinase (Cdk) family of proteins functions to regulate the cell cycle by promoting passage through cell cycle checkpoints. DNA damage leads to inhibition of Cdks, which allows cells to undergo cell cycle arrest and repair. The Cdc25 protein family can re-activates Cdks and allows re-entry into the cell cycle. MiR-21 has been shown to directly target Cdc25 in cancer cell lines and miR-21 inhibitors can enhance apoptosis in glioblastoma cells treated with ionizing radiation, suggesting a potential role of miR-21 as a radiosensitizer in these cells (Wang et al. 2009; Li et al. 2011).

6.4.2.4 Regulation of Repair Process

Finally, repair of DSBs ultimately occurs through two mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). In HR, the repair proteins use undamaged sister chromatid as a template to reconstruct the damaged region, and in NHEJ, the repair proteins simply rejoin the DNA fragments. MicroRNA regulation occurs in both of these mechanisms. For example, miR-182 has been reported to target HR protein breast cancer 1 (BRCA1). Overexpression of miR-182 leads to decreased HR-mediated DNA repair and renders the cells hypersensitive to ionizing radiation in breast cancer cells; thus revealing a potential role as a radiosensitizer (Moskwa et al. 2011).

6.5 Therapeutic Influence and Future Perspective

Taken together, the available literature indicates that microRNAs serve complex, diverse and sometimes seemingly contradictory regulatory roles in stress response signalling pathways in cancer. Through processes still not fully understood,

microRNAs are able to exert cell-specific and sometimes environment-specific effects. Nonetheless, these small molecules seem to serve an important role in maintaining homeostasis acting as buffers to balance signaling networks and thus show great therapeutic promise. MicroRNA-based therapies are progressing, and Miravirsen, an inhibitor of the liver-specific miR-122, is already in Phase IIa trials for the treatment of hepatitis C (Gebert et al. 2014).

MicroRNA profiling is a promising therapeutic application of these small molecules. Microarray analysis identifies those microRNAs expressed aberrantly in certain conditions, generating diagnostic and prognostic information which may allow the development of therapeutics tailored to the personal needs of specific patients. Since cancer represents a largely heterogeneous group of diseases, personalized medicine-based approaches are receiving increased attention.

Finally, microRNAs may be exploited for interventions that exogenously restore balance to abnormal signaling networks. However, the cell-line specific effects of microRNAs make this a challenging goal. Thus, advances in targeted delivery will be needed to truly facilitate the therapeutic use of microRNAs in the treatment of diseases such as cancer. Targeted delivery of microRNAs or microRNA inhibitors with chemosensitizing and radiosensitizing may lead to better management of the disease and lead to decreased cancer-related mortality.

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Chapter 7
Senescence in Oncogenesis: From Molecular Mechanisms to Therapeutic Opportunities

Sandra Muñoz-Galvan and Amancio Carnero

Abstract Somatic non stem cells show a spontaneous decline in growth rate in continuous culture related to an increasing number of population doublings, eventually terminating in a quiescent but viable state now known as replicative senescence. These cells show clear and distinctive morphological, physiological and biochemical characteristics. Moreover, the senescent phenotype is associated with a typical gene-expression profile. Similar behaviour has since then been observed in a wide variety of normal cells, and it is now widely accepted that normal somatic cells have an intrinsically limited proliferative lifespan, even under ideal growth conditions. Cells displaying characteristics of senescent cells, however, can be also observed in response to other stimuli, such as oncogenic stress, DNA damage or cytotoxic drugs. These non-proliferative characteristics prompted the scientists to look for therapies that can induce the senescent phenotype in tumor cells as therapeutic approach.

Keywords Cellular senescence • Immortalization • Cancer • Geroconversion • Telomere shortening • DNA methylation • Oncogenes • Telomerase • p53 • Retinoblastoma pathway • p16INK4a • Senescence clock • Senescence based therapy • Cell cycle

7.1 The Biology of Cellular Senescence

Over 40 years ago, Hayflick (1965) established that human diploid fibroblasts show a spontaneous decline in growth rate in continuous culture related not to elapsed time but to an increasing number of population doublings, eventually terminating in a quiescent but viable state now known as **replicative senescence**. These cells show clear and distinctive characteristics. Similar behaviour has since then been observed in a wide variety of normal cells, and it is now widely accepted (Hanahan and Weinberg 2000) that normal somatic cells have an intrinsically limited proliferative

S. Muñoz-Galvan • A. Carnero (✉)
Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocio,
Consejo Superior de Investigaciones Cientificas (CSIC), Universidad de Sevilla,
Avda. Manuel Siurot s/n, 41013 Sevilla, Spain
e-mail: acarnero-ibis@us.es

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lifespan, even under ideal growth conditions. Moreover, the senescent phenotype is associated with a typical gene-expression profile (Mason et al. 2004; Schwarze et al. 2005; Shay and Roninson 2004; Untergasser et al. 2002).

Cells displaying senescent characteristics have not only been observed in cell culture, but also in their maternal tissue environment. A number of reports have related reduced cellular lifespan with metabolic disease, stress sensitivity, progeria syndromes and impaired healing, indicating that entry into cellular senescence may contribute to human disease. Indeed, it has been suggested that cellular senescence is in part responsible for the pathogenesis of a number of human diseases, such as atherosclerosis, osteoarthritis, muscular degeneration, ulcer formation, Alzheimer's dementia, diabetes and immune exhaustion. Recently, a physiological role for senescence in embryonic development has been also uncovered (Munoz-Espin et al. 2013; Storer et al. 2013). However, most cancers contain cell populations that have escaped the normal limitations on proliferative potential. This capability, known as **immortality**, contrasts with the limited lifespan of normal somatic cells. It has therefore been proposed that cellular senescence is a major tumor suppressor mechanism that must be overcome during tumorigenesis (Hanahan and Weinberg 2000).

The kinetic of replicative senescence does not show an abrupt arrest of the whole population, but a gradual decline in the proportion of dividing cells (Thomas et al. 1997), the exact timing of which varies between both cell types and sister clones (Rubin 2002). This behaviour is best explained as the result of (i) an intrinsic control mechanism linked to elapsed cell divisions – **the senescence clock** – which progressively desensitises the cell-cycle machinery to growth factor stimulation, together with (ii) a stochastic component probably having the same (still unknown) basis as that observed in immortal cells under conditions of growth factor restriction. Stem cells can give rise to differentiated progeny and are capable of auto-renewal. In some renewing tissues, stem cells undergo more than 1,000 divisions in a lifetime with no morphological signs of senescence (Rubin 2002). This indicates that at a certain point of lineage differentiation cells activate the senescence clock that ultimately induces cell senescence through a series of effectors.

In humans, the finite number of divisions – referred to as the '**Hayflick limit**' – was attributed to the progressive shortening of chromosomal ends. **Telomere shortening** is considered to be the most probable molecular mechanism explaining the existence of such a senescence clock controlling replicative senescence (Kipling et al. 1999; Wright and Shay 1995). Eukaryotic cells cannot replicate the very ends of their chromosomes, the telomeres, resulting in a shortening their lengths with every cell division until they reach a critical threshold, at which point cells stop replicating (Olovnikov 1973). However, enforced replication despite short telomeres ends in high chromosomal instability and apoptosis, a process known as **crisis**. Many other mechanisms, however, have been also proposed (Vergel et al. 2010). In this context it is essential to clarify major differences between early passage human and rodent cells with respect to the senescence barriers that need to be bypassed to achieve full immortalization. Cells from small rodents (mice, rats, hamster) have a single barrier to immortalization, that can be readily bypassed via pRB pathway (mutational or epigenetic) or p53 (mutational) pathways inactivation. Human cells (fibroblasts and

variety epithelial cells) require, in addition, bypass of telomere-driven replicative senescence through reactivation of telomerase (transcriptional derepression of hTERT) an extremely rare event. The differences originates from the fact that rodent cells have telomerase permanently 'on' even when irreversibly senescent (Russo et al. 1998).

Senescent features involve most of the physiological aspects of the cell. Morphologically senescent cells show flat, enlarged morphology and are commonly multinucleated (Pospelova et al. 2013). Senescent cells are terminally arrested at G1, showing increased levels of many cell cycle inhibitors (Zhao and Darzynkiewicz 2013). They show, of course, short telomeres. The telomerase gene is deactivated in most adult human cells. As a result, these cells lose small portions of the ends (telomeres) of their chromosomes each time they divide. This process appears linked to their finite replicative lifespan in cell culture (The Hayflick Limit). However, oncogene- or culture stress-induced senescence does not rely on telomere shortening (Maritz et al. 2013).

Senescent cells show altered lysosome/vacuoles. The recycling centers inside of cells are lysosomes. Abnormal chemical structures, which resist degradation, accumulate in the lysosomes during the lifespan of the cells or during stress-induced senescence. The result is the eradication of lysosomal recycling capacity for proteins, lipids and mitochondria. Consequently, damaged mitochondria accumulate in these cells, which lower ATP production and elevate ROS production. Furthermore, oxidative damaged enzymes accumulate in the cytosol, which reduces the rate of essential cellular functions. It is thought that the Senescence-associated β-galactosidase (SA-β-gal) activity, which is detected by histochemical staining of cells with the artificial substrate X-gal, is dependent on the altered lysosomal content. The presence of the SA-β-gal biomarker is independent of DNA synthesis and generally distinguishes senescent cells from quiescent cells (Bassaneze et al. 2013; Itahana et al. 2013).

The genomic methylation status, which influences many cellular processes, such as gene expression and chromatin organization, generally declines during cellular senescence. Hypomethylation has been observed in both replicative senescence and premature senescence, which suggests that genome hypomethylation is necessary to confer an unstable internal environment and conceivably promote cellular senescence (Carnero and Lleonart 2011; Zhang et al. 2008).

The initiation of senescence triggers the generation and accumulation of distinct heterochromatic structures, known as senescence-associated heterochromatic foci (SAHF) (Fig. 7.1). The formation of SAHF coincides with the recruitment of heterochromatic proteins and the pRb tumor suppressor to E2F-responsive promoters. SAHF accumulation is associated with stable repression of E2F target genes and does not occur in reversibly arrested cells. SAHF formation and promoter repression depend on the integrity of the pRb pathway (Narita et al. 2003). These results provide an explanation for the stability of the senescent state. Accordingly, with these results, genome-wide expression analysis indicates that genes whose expression is upregulated during replicative senescence in human cells are physically clustered (Zhang et al. 2003). This phenomenon suggests that senescence is accompanied

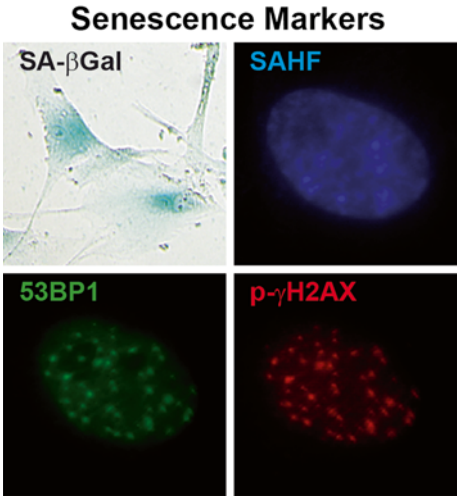

Fig. 7.1 Molecular markers of senescence. Pictures showing some examples of senescence molecular markers, including senescence-associated b-galactosidase (SA-bGal) activity; senescence-associated heterochromatin foci (SAHF), visualized by immunofluorescence microscopy with DAPI staining; and two senescence-associated DNA damage markers: 53BP1 and the phosphorylated histone H2A variant gH2AX (p- gH2AX), visualized by immunofluorescence microscopy using specific antibodies

by alterations in chromatin structure and the opening of certain chromatin domains is responsible for the concurrent upregulation of gene expression during senescence (Young and Narita 2013).

Senescent cells display molecular characteristics of DNA damage (Fig. 7.1). Markers of a DNA damage response localize at telomeres in senescent cells after serial passage (d'Adda di Fagagna et al. 2003; d'Adda di Fagagna 2008; Herbig et al. 2004), which indicates that the DNA damage response can be triggered by telomere shortening. These markers include nuclear foci of phosphorylated histone H2AX, the localization at double-strand break sites of DNA-repair and DNA-damage checkpoint factors, such as 53BP1, MDC1 and NBS1 (d'Adda di Fagagna 2008; Ruiz et al. 2008). Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. These markers and others suggest that telomere shortening initiates senescence through a DNA damage response. These characteristics also explain why other DNA damage stressors, such as culture shock, potentially initiate senescence without telomere involvement (Hewitt et al. 2013). In parallel, the redox potential poise of some cells changes in response to chemical modifications. This modification results in altered gene expression, enzyme activity, and signaling pathways. Finally, it results in DNA damage (Nelson and von Zglinicki 2013; Passos et al. 2013).

Large protein and lipid modification is another characteristic of senescent cells (Gasparovic et al. 2013; Vistoli et al. 2013). Oxidation, glycation, cross-linking, and other chemical modifications impair the molecular functions of multiple vital

components, including DNA, membranes, the extracellular matrix (ECM), enzymes, and structural proteins. Modifications that accumulate faster than they are repaired or recycled will cause progressive deterioration over time.

Nuclear envelope alterations in senescence nuclear structures, such as the nuclear lamina, nucleoli, the nuclear matrix, nuclear bodies (such as promyelocytic leukemia bodies), and nuclear morphology are altered within growth-arrested or senescent cells. It is especially interesting that multinucleation is probably the consequence of the failure of nuclear envelope breakdown (de la Rosa et al. 2013).

Senescence-associated secretory phenotype (SASP). Senescent cells undergo widespread changes in protein expression and secretion, which ultimately develops into the SASP (Campisi 2011; Campisi et al. 2011). Senescent cells upregulate the expression and secretion of several matrix metalloproteinases that comprise a conserved genomic cluster and interleukins that promote the growth of premalignant epithelial cells. A limited number of cell culture and mouse xenograft studies support the idea that senescent cells secrete factors that can disrupt tissue structure, alter tissue function and promote cancer progression (Bavik et al. 2006; Krtolica et al. 2001; Parrinello et al. 2005). Recent studies on the SASP of human and mouse fibroblasts show it is conserved across cell types and species; moreover, specific secreted factors are strong candidates for stimulating malignant phenotypes in neighboring cells (Coppe et al. 2008, 2010a, b). The idea that a biological process, such as cellular senescence, can be beneficial (tumor suppressive) and deleterious (pro-tumorigenic) is consistent with a major evolutionary theory of aging termed antagonistic pleiotropy (Coppe et al. 2010a). The SASP is possibly the major reason for the deleterious side of the senescence response (Davalos et al. 2010; Rodier 2013).

Consistent with a role in aging, senescent cells accumulate with age in many rodent and human tissues (Campisi 2005). Moreover, they are found at sites of age-related pathology, including degenerative disorders such as osteoarthritis and atherosclerosis (Campisi 2005) and hyperproliferative lesions such as benign prostatic hyperplasia (Castro et al. 2003) and melanocytic naevi (Michaloglou et al. 2008). A limited number of cell culture and mouse xenograft studies support the idea that senescent cells secrete factors that can disrupt tissue structure and function and promote cancer progression (Bavik et al. 2006; Krtolica et al. 2001; Parrinello et al. 2005). Recent studies on the senescence-associated secretory phenotype (SASP) of human and mouse fibroblasts show it is conserved across cell types and species and that specific secreted factors are strong candidates for stimulating malignant phenotypes in neighboring cells (Coppe et al. 2008, 2010a, b).

The idea that a biological process such as cellular senescence can be both beneficial (tumor suppressive) and deleterious (pro-tumorigenic) is consistent with a major evolutionary theory of aging termed antagonistic pleiotropy (Coppe et al. 2010a). The SASP may be the major reason for the deleterious side of the senescence response (Davalos et al. 2010).

As mentioned, in addition to telomere dysfunction, cellular senescence can be elicited by other types of stress, including oncogene activation (Collado and Serrano 2006). This phenomenon is not observed for oncogenic RAS exclusively; many – but

not all – of its effectors, including activated mutants of RAF, MEK and BRAF, were shown to cause senescence as well (Braig and Schmitt 2006; Carnero et al. 2003; Courtois-Cox et al. 2008; Chandeck and Mooi 2010). Some oncogenes, such as RAS, CDC6, cyclin E, and STAT5, trigger a DNA damage response (DDR), associated with DNA hyper-replication that appears to be causally involved in oncogene-induced senescence (OIS) in vitro (Bartek et al. 2007; Di Micco et al. 2006; Kenyon and Gerson 2007; Ruzankina et al. 2008). During most of the last decade, OIS has been studied predominantly in cell culture systems, triggering a long debate as to whether or not OIS corresponds to a physiologically relevant phenomenon in vivo. In favour of OIS representing an in vitro phenomenon only is that artificial conditions, such as the use of bovine serum and plastic dishes, as well as the presence of supraphysiologic O2, generate a stress signal that at the very least contributes to triggering a cellular senescence response (Parrinello et al. 2003; Passos and Von Zglinicki 2006). However, conversely, senescence bypass screens have identified several genuine human oncogenes, including TBX2, BCL6, KLF4, hDRIL, BRF1, PPP1CA and others (Vergel and Carnero 2010). Furthermore, virtually all human cancers lack functional p53/pRB pathways, two key senescence- signalling routes (Malumbres and Carnero 2003), and often carry mutations in sets of genes, which are known to collaborate in vitro in bypassing the senescence response.

7.2 Effector Pathways

Cellular senescence pathways are believed to have multiple layers of regulation, with additional redundancy built into these layers (Smith and Pereira-Smith 1996). On the basis of the complementation studies, there are at least four senescence pathways (Duncan et al. 1993). This indicates that in any one immortal cell line, there are probably multiple senescence genes/pathways that are abrogated (Barrett et al. 1994). Many of the functional studies, where a putative senescence gene is overexpressed in cells, indicate that although multiple genes/pathways may be abrogated in a particular cell line, as little as one gene/pathway is required for repair and subsequent reversion to senescence, indicating that senescence is essentially a dominant phenomena (Fig. 7.2).

Pathways known to regulate cellular senescence/immortalisation, including the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1/WAF1 pathway and the PTEN/p27KIP1 pathway, are reviewed in (Carnero and Lleonart 2010; Mooi and Peeper 2006; Schmitt 2007; Serrano and Blasco 2001; Vergel and Carnero 2010). Other genes that have been shown to contribute to a senescence-like phenotype include PPP1A (Castro et al. 2008b), SAHH (Leal et al. 2008a: ME et al. 2006), Csn2, Arase and BRF1 (Leal et al. 2008b), PGM (Kondoh et al. 2005), IGFBP3 and IGFBPrP1 (Fridman et al. 2007), PAI-1 (Kortlever and Bernards 2006; Kortlever et al. 2006), MKK3 (Wang et al. 2002), MKK6 (Haq et al. 2002; Wang et al. 2002),Smurf2 (Zhang and Cohen 2004) and HIC-5 (Shibanuma et al. 1997). All these genes have shown to be related to human tumorigenesis. However, all these

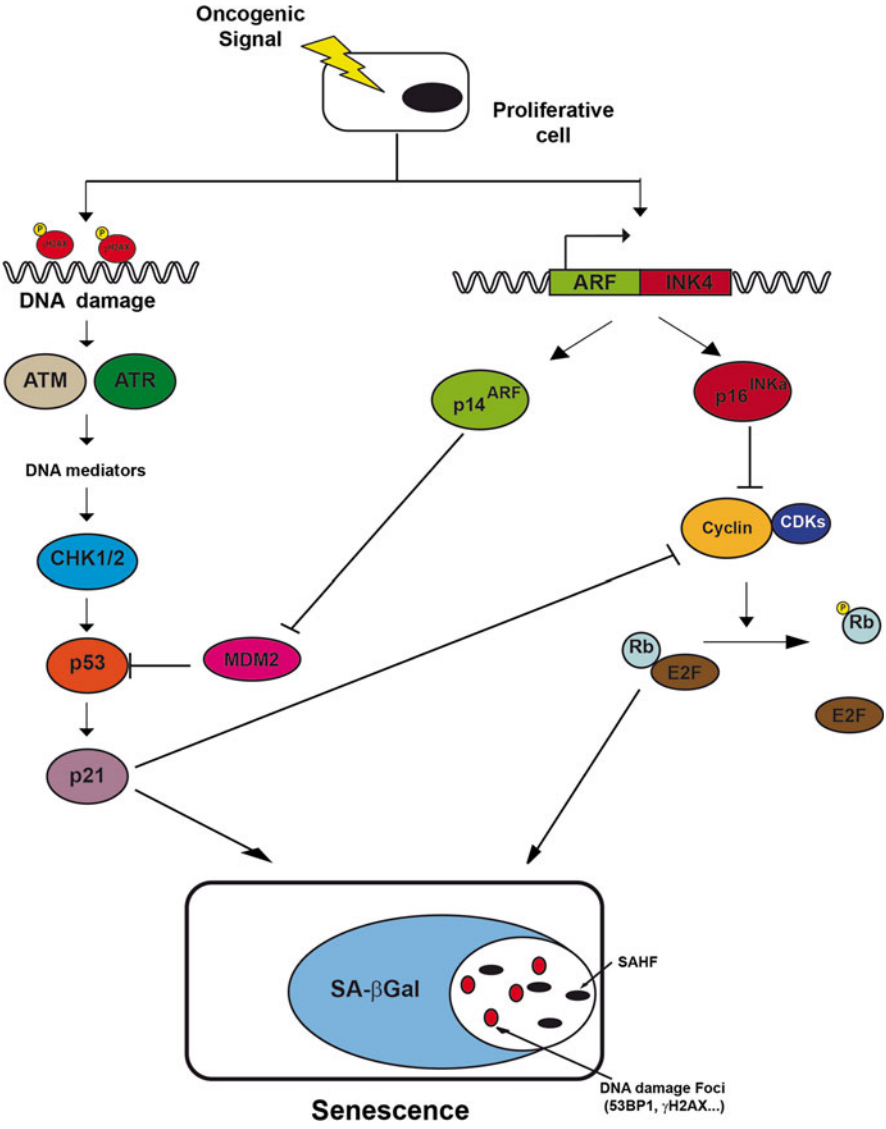

Fig. 7.2 Molecular pathways of oncogenic-induced senescence. When a proliferative cell suffers oncogenic stress, two different pathways leading to cellular senescence are activated: one p53-dependent, induced by DNA damage checkpoint activation, and other p16-Rb-dependent, which stops cell cycle progression. Both pathways interact functionally giving some redundancy to the effector signal

genes and their pathways, as indicated earlier, can act in sequential steps conforming a well-regulated process.

The dynamics of senescence exhibit two different steps: cell cycle arrest and further acquisition of senescence features, which includes permanent arrest, termed **geroconversion**.

Senescence effector pathways converge at the point of cell cycle arrest through CDK inhibition. Therefore, most pathways known to be involved in senescent arrest impinge either directly or indirectly on this process. Namely, the most known effector pathways are the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1 pathway and the PI3K/mTOR/FoxO pathway (Carnero and Lleonart 2010; Mooi and Peeper 2006; Schmitt 2007; Serrano and Blasco 2001; Vergel and Carnero 2010), all of which exhibit a high degree of interconnection. Two pathways have been proposed to be responsible for the acquisition of irreversible arrest and geroconversion: the pRB pathway and the mTOR pathway. If geroconversion is not activated, cells are only transiently arrested with the possibility of resuming growth once the proliferation constraints have been eliminated (Ferbeyre et al. 2002; Ruiz et al. 2008). It has also been shown that if mTOR is activated under conditions of proliferative arrest, then arrest becomes permanent and the cell undergoes senescence (Blagosklonny 2010; Demidenko and Blagosklonny 2008). This can also be accomplished by producing permanent changes in the chromatin, especially at E2F transcription sites, which results in a blockade of transcription of proliferative genes (Narita et al. 2003). It has been shown that permanent inactivation of pRb, perhaps in combination with phosphatases (Castro et al. 2008a), may signal for the differential recruitment of silencers to the heterochromatin of promoter sites. Human cells show heterochromatin compaction during senescence (SAHF for senescence-associated heterochromatin foci), which is dependent on the pRb pathway (Narita and Lowe 2004). These SAHFs cause stable silencing of cell cycle genes and seem to be a factor in the stability of permanent arrest during senescence. Also, the role of senescence in embryonic development seems to be dependent on the pRb pathway through CDK inhibitors p21CIP1 and p15INK4b but independent of other cell cycle inhibitors, DNA damage or p53. This senescence during embryonic development seems also regulated by the PI3K/FOXO and TGFb/SMAD pathways (Munoz-Espin et al. 2013; Storer et al. 2013).

The absence of **p53 function** induced by dominant negative mutants, specific p53 antisense, oligonucleotides or viral oncoproteins (such as SV40 T antigen or HPV16 E6) is sufficient to substantially extend the lifespan of several cell types in culture (Wynford-Thomas 1996). Consistent with this, senescence is associated with a switch-on of the transactivation function of p53 in culture (Bond et al. 1996). Coincident with telomere shortening, DNA-damage checkpoint activation and associated genomic instability, p53 is also activated in vivo (Chin et al. 1999). Deletion of p53 attenuated the cellular and organismal effects of telomere dysfunction, establishing a key role for p53 in the shortening response (Chin et al. 1999). Other p53 regulatory proteins are also involved in senescence. MDM2 protein has p53 ubiquitin ligase activity and forms an autoregulatory loop with p53 (Ashcroft et al. 2000). Overexpression of MDM2 targets p53 for degradation and induces functional-p53 loss (Blaydes and Wynford-Thomas 1998). The product of another

gene up-regulated in senescence – p14ARF – can release p53 from inhibition by MDM2 and cause growth arrest in young fibroblasts (Blaydes and Wynford-Thomas 1998). Seeding mouse embryonic fibroblasts (MEFs) into culture induces the synthesis of ARF protein, which continues to accumulate until the cells enter senescence (Kamijo et al. 1997). MEFs derived from ARF-disrupted mice (Kamijo et al. 1997) or wild type fibroblasts expressing an efficient ARF antisense construct (Carnero et al. 2000b) are also efficiently immortalised. Concomitant with this observation, overexpression of MDM2 in naïve MEFs produces efficient immortalisation (Carnero et al. 2000b). Activation of p53 induces the up-regulation of the cyclin-dependent kinase (CDK) inhibitor p21WAF1, which has a direct inhibitory action on the cell-cycle machinery (Malumbres and Carnero 2003) and correlates well with the declining growth rate in senescing cultures. In mouse embryo fibroblasts however, the absence of p21WAF1 does not overcome senescence (Carnero and Beach 2004; Pantoja and Serrano 1999). This suggests that at least one additional downstream effector is needed for p53-induced growth arrest in senescence. In contrast, a different behaviour is observed in human cells, where elimination of p21 by a double round of homologous recombination is sufficient to bypass senescence (Brown et al. 1997). Other p53 effectors might be also involved, such as 14-3-3 and GADD45, which inhibit G2/M transition, or downregulation of Myc (Ho et al. 2005).

The **retinoblastoma tumor suppressor pathway**, pRb, has also been related to senescence. Overexpression of pRb, as well as some of the regulators of the pRb pathway such as CDK inhibitors, leads to growth arrest mimicking the senescent phenotype (Carnero et al. 2003). Moreover, inactivation of pRb by viral oncoproteins such as E7, SV40 large T antigen and E1A leads to extension of lifespan (Haferkamp et al. 2009; Jarrard et al. 1999; Ye et al. 2007). Other members of the pocket protein family comprising pRb, p130 and p107 may also be involved. In MEFs, p130 levels decrease with population doublings and MEFs from triple pRb, p130 and p107 knockout mice are immortal (Mulligan and Jacks 1998). Nevertheless, since a certain degree of complementation has been observed among the pocket protein family (Mulligan and Jacks 1998), it is difficult to assess the role of each protein in replicative senescence.

Given that p16INK4a functions to inhibit the inactivation of pRb by CDKs (Carnero and Hannon 1998), a loss of functional p16INK4a may be expected to have similar consequences with the loss of functional pRb. Several types of human cells accumulate p16INK4a protein as they approach senescence (Palmero et al. 1997). Senescent fibroblasts may contain p16INK4a levels at least 40-fold greater than early passage cells. The deletion of p16INK4a is common in immortalised tumor cell lines (Okamoto et al. 1994), and several non-tumorigenic in vitro immortalised cell lines also lack functional p16INK4a protein. Expression of p16INK4a-specific antisense in naïve MEFs increases the probability of immortalisation of these cells (Carnero et al. 2000b). In accordance with this observation, mice cells which are made nullizygous for p16INK4a by targeted deletion undergo immortalisation more readily than normal control cells (Krimpenfort et al. 2001; Sharpless et al. 2001), although they show normal

senescence kinetics. Knockout mice for p16INK4a proteins develop normally to adulthood and are fertile, indicating that the individual INK4 proteins are not essential for development. p16INK4a deficiency, however, results in a low susceptibility to spontaneous tumor development and increased tumor susceptibility under specific carcinogenic protocols (Krimpenfort et al. 2001; Sharpless et al. 2001). A cross-talk among the different pathways involved in senescence has been found. This cross-talk might ensure the correct functioning of the senescence program. Moreover, genes such as *myc* that are involved in all the pathways are able to bypass senescence in human primary cells. Myc can bypass CDK4/6 inhibition by activating CDK2-cyclinA/E complexes and inducing the Cdk-activating phosphatase Cdc25A (Amati et al. 1998). Moreover, *myc* induces degradation of p27, thus influencing the inhibitory effects of PTEN. Finally, expression of myc induces telomerase activity by activating the transcription of the catalytic subunit (Wang et al. 1998a). The overall result is a single step immortalisation of human cells induced by myc gene amplification (Gil et al. 2005).

PI3K/AKT/mTOR/FoxO constitutes an important pathway regulating the signaling cascades of multiple essential biological processes (Blanco-Aparicio et al. 2007; Carnero 2010; Lacal and Carnero 1994). Many components of this pathway are genetically altered in cancer cells. AKT is a master kinase that phosphorylates MDM2 (among other proteins) and promotes its translocation to the nucleus, where it negatively regulates p53 function (Gottlieb et al. 2002). One of the most conserved functions of AKT is its role in cell mass increase through the activation of the mTOR complex 1 (mTORC1 or the mTOR/raptor complex), which is regulated by both nutrients and growth factor signaling. mTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control (Sengupta et al. 2010). PI3K has been related to the induction of cellular senescence in several ways that are still not fully understood. Early works from Collado and colleagues, (Collado et al. 2000) suggest that PI3K inhibition induces senescence through the activation of p27kip1. However, further works also indicated that the overexpression of active P110a (catalytic subunit of PI3K) or AKT induces oncogene-induced senescence in primary cells in culture and in vivo (Carnero and Beach 2004; Di Cristofano et al. 2001; Lorenzini et al. 2002; Renner and Carnero 2009; Trotman et al. 2006). On the other hand, loss of PTEN triggers cellular senescence through a p53-dependent mechanism (Chen et al. 2005) and results in indolent prostate cancer. Therefore, concomitant or sequential loss of PTEN and p53 results in a dramatic acceleration of prostate tumorigenesis. Studies in murine mouse models have shown that p53 is the preferred mutation upon PTEN loss. In constitutively active AKT or PI3K transgenic models, an increase in benign lesions are observed if senescence is induced upon AKT activation (Blanco-Aparicio et al. 2010; Renner et al. 2008).

AKT activation can also stimulate proliferation through multiple downstream targets and impinge on cell-cycle regulation. AKT phosphorylates some members of the FoxO family while they are present in the nucleus, thus creating binding sites for 14-3-3-sigma proteins that trigger their export from the nucleus. Through this mechanism, AKT blocks the FoxO-mediated transcription of target genes that

promote apoptosis, cell-cycle arrest, and metabolic processes (Calnan and Brunet 2008; Zanella et al. 2010).

FoxO transcription factors are an evolutionary conserved subfamily that regulate a number of cellular processes involved in cell-fate decisions in a cell-type- and environment-specific manner, including metabolism, differentiation, apoptosis and proliferation (Greer and Brunet 2008). A key mechanism by which FoxO determines cell fate is through regulation of the cell cycle machinery. FoxO plays a crucial role in regulating cellular senescence by controlling the expression of a number of cell cycle regulators, including p27kip1 (Collado et al. 2000). Moreover, overexpression of FoxO or p27KIP1 in primary mouse embryo fibroblasts can recapitulate this phenotype, promoting premature cell cycle arrest, changes in cell morphology and increases in senescence-associated markers. The ability of FoxO to induce G0/G1 arrest is lessened in p27Kip1 and p130 double deficient fibroblasts (Chen et al. 2006), suggesting that both p27Kip1 and p130 are important for mediating FoxO-dependent cellular senescence associated G0/G1 arrest. Further evidence of a role for FoxO in cellular senescence is supported by a recent in vivo study demonstrating that oncogene-induced senescence also involves the repression of the PI3K–PKB signaling pathway and the induction of FoxO (Kyoung Kim et al. 2005).

mTOR is an essential convergence point for the PI3K/AKT/FoxO pathways (Martinez-Gac et al. 2004). mTOR is the master regulator of protein synthesis (Wullschleger et al. 2006). It has been proposed that for growth arrest to become permanent (i.e., undergo senescence), a high level of mTOR activation is necessary (Blagosklonny 2008, 2009). In fact, rapamycin treatment, which inhibits mTOR, can divert senescence into quiescence, allowing the cell to resume growth once conditions are more favorable (Anisimov et al. 2010; Korotchkina et al. 2010). It has been proposed that this contribution is due to the function of mTOR as a sensor of cellular nutrients and energy status as well as growth factor signals. mTOR then integrates those signals and "decides" whether the amount of metabolites and energy are sufficient to permit protein synthesis (Sengupta et al. 2010; Young and Narita 2013).

Crosstalk among the different pathways involved in senescence has been found (Carnero et al. 2000b). This crosstalk might ensure the accurate execution of the senescence program. Moreover, genes, such as *myc,* that are involved in these pathways bypass senescence in human primary cells. Myc can bypass CDK4/6 inhibition by activating CDK2-cyclinA/E complexes and inducing the Cdk-activating phosphatase Cdc25A (Amati et al. 1998). Moreover, *myc* induces degradation of p27, which influences the inhibitory effects of PTEN. Finally, expression of myc induces telomerase activity by activating the transcription of the catalytic subunit (Wang et al. 1998a). The overall result is a single step immortalization of human cells induced by myc gene amplification (Gil et al. 2005).

MicroRNAs (miRNAs) are small non-coding endogenous RNA molecules that regulate gene expression and protein coding by base pairing with the 3′ untranslated region (UTR) of target mRNAs. MiRNA expression is associated with cancer pathogenesis because miRNAs are intimately linked to cancer development. Senescence blocks cell proliferation and represents an important barrier that cells

must bypass to reach malignancy. Importantly, certain miRNAs have an important role during cellular senescence, which is also involved in human tumorigenesis (Feliciano et al. 2011).

Several miRNAs are differentially expressed in senescent cells when compared to primary cells, which implies a role for miRNAs in senescence. Recently, miR-34a overexpression has been reported during senescence and can cause senescence in a p53-independent manner through repression of c-myc (Christoffersen et al. 2010). MiR-34a is downregulated in pancreatic cancer cells, neuroblastomas, colon cancer cells, and lung cancer cells (Bommer et al. 2007; Tazawa et al. 2007), which suggests a mechanism for immortalization. The expression levels of miR-29 and miR-30 increase during cellular senescence, and these microRNAs directly repress B-Myb in conjunction with Rb-E2F complexes, which results in senescence (Martinez et al. 2011). MiR-29 is downregulated in cell lymphomas (Zhao et al. 2010), and the overexpression of miR-29 is suppressed during tumorigenicity in lung cancer cells (Fabbri et al. 2007). MiR-449a suppresses pRb phosphorylation, which induces senescence (Marasa et al. 2010; Noonan et al. 2009, 2010). A recent study has shown that miR-449a is downregulated in prostate cancer, which indicates that this miRNA regulates cell growth and viability, in part by repressing the expression of HDAC-1 (Noonan et al. 2009). MiR-128a directly targets the Bmi-1 oncogene (polycomb ring finger oncogene; BMI1), which increases p16INK4A expression and reactive oxygen species (ROS). Collectively, these effects promote cellular senescence in medulloblastoma cell lines. MiR-217, which is expressed in endothelial cells during aging, promotes premature senescence by inhibiting SIRT1 expression. This occurrence increases forkhead box O1 (FoxO1) expression (Garzon et al. 2008). In addition, miR-217 has been reported to be a novel tumor suppressive miRNA that targets K-Ras in pancreatic ductal adenocarcinoma due to decreases in tumor cell growth both in vitro and in vivo (Menghini et al. 2009). MiR-20a induces senescence in MEFs by directly downregulating the transcriptional regulator leukemia/lymphoma-related factor (LRF), which induces p19ARF (Borgdorff et al. 2010). In addition, miR-519 induces senescence in cancer cell lines by repressing HuR expression (Voorhoeve et al. 2006). In contrast, there are miRNAs that are downregulated during senescence, such as miR-15b, miR-24, miR-25, and miR-141, which directly target mitogen-activated protein kinase kinase (MKK4) (Cho et al. 2009). Recently, it was shown that 28 miRNAs prevented senescence induced by oncogenic RasG12V (Borgdorff et al. 2010). These miRNAs bypass RasG12V-induced senescence by directly targeting the 3′UTR of p21Cip1. Moreover, miR-372, miR-373, miR-302, and miR-520 also bypass RasG12V-induced senescence through the downregulation of LATS2 in addition to p21Cip1 (Borgdorff et al. 2010). These identified proliferative miRNAs are associated with cancer development (Leal et al. 2013; Feliciano et al. 2011).

Over all steps, **DNA methylation** regulates expression of senescence genes, with the capability of controlling the process (Carnero and Lleonart 2010). In human cancers, the silencing of tumour suppressor genes through aberrant DNA methylation of the CpG island(s) in promoters in these genes is a common epigenetic change (Baylin et al. 2000). There are an assortment of pathways from which genes have been shown

to be hypermethylated in cancer cells, including DNA repair, cell-cycle control, invasion and metastasis. The tumour suppressor genes BRCA1, p16INK4a, p15INK4b, p14ARF, p73 and APC are among those silenced by hypermethylation, although the frequency of aberrant methylation is somewhat tumour-type specific. Recently, we found S-adenosylhomocysteine hydrolase (SAHH) (Leal et al. 2008a), which has also been previously identified in an independent short hairpin RNA (shRNA) screening (Brummelkamp et al. 2004), the inactivation of which confers resistance to both p53- and p16(INK4)-induced proliferation arrest and senescence. SAHH catalyzes the hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine. In eukaryotes, this is the major route for disposal of S-adenosylhomocysteine formed as a common product of each of the many S-adenosylmethionine-dependent methyltransferases, therefore regulating the methylation processes. Interestingly, SAHH inactivation inhibits p53 transcriptional activity and impairs DNA-damage-induced transcription of p21(Cip1). SAHH messenger RNA (mRNA) was lost in 50 % of tumour tissues from 206 patients with different kinds of tumours in comparison with normal tissue counterparts. Moreover, SAHH protein was also affected in some colon cancers (Leal et al. 2008a; Castro et al. 2006).

7.3 Clinical Implications

The implication of senescence as a barrier to tumorigenesis first comes from the realisation that a limited number of duplications necessarily reduces the possibility of tumor growth. However, the proliferative lifespan before reaching the Hayflick limit could be sufficient to generate a tumor mass greater than that required for lethality. This argument fails to take into account the existence of ongoing cell death and differentiation within a tumor and the occurrence of clonal selection driven by different senescence barriers or barriers unrelated to senescence. Finally, a clinically significant cancer can be composed of entirely mortal, pre-senescent cells if the cell of origin has a sufficient proliferative lifespan and the tumor develops with few successive clonal expansion steps and/or with a low cell death rate. Even with these examples, however, senescence may of course still be a significant barrier to the recurrence of tumors from the small number of residual cells remaining after therapy.

As mentioned, several studies in vivo show that oncogene-induced senescence provides a bona-fide barrier to tumorigenesis. Michaloglou and co-workers (Michaloglou et al. 2005) have shown that an oncogenic BRaf can induce senescence in fibroblasts and melanocytes and that human nevi display markers of senescence. Therefore, sustained exposure of melanocytes to aberrant mitotic stimuli provokes senescence after an initial proliferation burst. Collado and co-workers (Collado et al. 2005) identified senescent cells in vivo after generating new senescence biomarkers from array studies. Using conditional Kras-val12 mice strains they observed senescence markers to be predominant in premalignant lesions of the lung and pancreas, but not in those that have progressed to full-blown cancers.

Direct evidence that hyperproliferative signals can trigger a program of permanent arrest in vivo have been provided in a transgenic model conditionally expressing E2F3 in the pituitary gland (Lazzerini Denchi et al. 2005). E2F3 induced hyperplasias that failed to progress because the cells became insensitive to further mitogenic signals. This insensitivity correlated with the appearance of senescence markers and a terminally arrested cellular state. Disruption of PTEN in mice also produces hyperplastic conditions analogous to prostatic intraepithelial neoplasia (a precancerous lesion in men). These lesions display senescence markers (Chen et al. 2005). Loss of p53 prevents senescence in response to PTEN ablation and cooperates to produce invasive prostate carcinomas. These results are consistent with the notion that senescence actively limits malignant conversion.

In human fibroblasts in culture, the senescence program involves chromatin reorganisation involving H3 methylation at the Lys9 residue concomitant with the recruitment of heterochromatin proteins to some proliferation-related genes. Braig and co-workers (Braig et al. 2005) found that disruption of Suv39h1 methyltransferase, which methylates the Lys9 residue of H3, blocked ras-induced senescence and accelerated ras-induced lymphomagenesis in mice. Interestingly, Suv39h1-expressing tumors responded through senescence to chemotherapy; however, Suv39h1-null tumors did not show any senescent response but still maintained the apoptotic response. Treating ras transgenic mice with DNA-methyltransferase or histone deacetylase inhibitors, which mimic the effects of Suv39h1 disruption, accelerated ras-induced tumorigenesis.

The concept of cancer being a disease whereby cells have lost the ability to senesce leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis and therapy through the knowledge surrounding molecular pathways (both genetic and epigenetic in origin) that induce senescence. Until just a few years ago, it was accepted that tumor cells were no longer capable of senescence. Today, however, it is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation and differentiating agents (Carnero et al. 2003; Roninson 2003). However, although not fully studied in vivo, it has been shown that senescent cells might increase the oncogenic potential of tumor cells. Therefore it will be necessary to understand the contribution of senescent stromal cells to tumors, before applying drug-induced senescence program to tumors.

Immortalising defects are recessive and can be blocked by imposing the process of senescence (Pereira-Smith and Smith 1988). The first approach to inducing senescence to tumor cells was through somatic cell fusion. These studies identified four senescence-determining complementation groups. In recent years, it has been found that different tumoral cell lines show cellular growth arrest along with senescence markers after the genetic expression of tumor suppressor genes commonly involved in senescence, such as p53, p21, p16, pRb or p21 (McConnell et al. 1998). Similarly, the restoration of cellular levels of p53 in a cell line conditionally immortalised by p53 antisense expression induces growth arrest with a senescent phenotype (Carnero et al. 2000a). Adenovirus vectors carrying CKIs (p16INK4a, p15INK4b, p21cip1 and p27kip1) as vehicles for delivery and

expression are a powerful approach to examining therapeutic applications both in vitro and in vivo, with promising results (Carnero 2002). When a 16-amino acid transmembrane carrier segment derived from the *Drosophila* antenappedia protein was linked to the third ankyrin repeat of the p16INK4a protein and inserted into cells, Rb-dependent G_1 arrest was observed. In a breast-derived cell line, the chimera containing the antennapedia peptide and the carboxyl-terminal residue of p21waf1 had higher specificity for CDK4/cyclin D than for CDK2/cyclin E and arrested the cells in G_1 phase (Ball et al. 1997).

These observations indicate that tumor cells maintain at least some of the components of the cellular senescence program, including terminal growth arrest. It is now clear that, depending upon the cell proliferation kinetics of the tissue of origin, tumor development can be initiated by genetic events, causing either a block in terminal differentiation or/and inappropriate activation of growth stimulatory signaling pathways. The net result in both cases is the generation of a cellular clone capable of infinite expansion if it is not constrained by physical barriers or lack of blood supply. Lowe and collaborators (Schmitt et al. 2002) convincingly showed that in a lymphoid mouse tumor model, an intact senescence pathway appears to be pivotal to the efficacy of cyclophosphamide, and its disruption makes tumor cells highly refractory to the drug. On the other hand, as mentioned, Suv39h1-expressing tumors responded to chemotherapy by inducing senescence. However, Suv39h1- null tumors did not show any senescent response but still maintained the apoptotic response. Suv39h1-null tumors with altered apoptotic response do not respond to therapy.

These results suggest that drug efficacy and tumor formation are not fully independent processes. Until recently, tumor formation and the development of drug resistance were thought to be independent processes. Mutations in factors that regulate tumor-suppressive fail-safe mechanisms, such as apoptosis and senescence, allow transformation. Chemotherapeutic compounds activate a separate set of effector pathways that eliminate malignant clones. Mutations in factors that are involved in these separate pathways inhibit the effect of chemotherapy to induce the effector programs to eliminate the tumors. Consequently, defects in antineoplastic fail-safe programs, even if required to allow for tumor formation, do not interfere with the effector program initiated by therapeutic agents. Nevertheless, preclinical data have provided evidence that key regulators, such as p53, participate in tumor prevention and drug action, and that tumor mutations acquired during tumor development also confer chemoresistance (Lowe et al. 1993, 2004).

The in vitro observation that DNA-damaging agents not only promote apoptosis but also induce cellular senescence (Roninson 2002; te Poele et al. 2002) indicates that genes that control senescence might also determine treatment outcome. Using a MYC-driven mouse lymphoma model, p53 and p16INK4A were recently shown to control drug-induced senescence in vivo (Schmitt et al. 2002). Drug-treated lymphomas with apoptotic defects were forced into senescence, and tumors that resumed growth frequently displayed defects in either p53 or p16INK4A. Importantly, drug-induced senescence was shown to contribute to long-term host survival after cancer therapy, as mice bearing lymphomas that were unable to enter senescence in response to therapy had shorter survival times. Notably, drug-inducible senescence

is not a phenomenon that is restricted to a mouse lymphoma model, as tissue specimens taken from human breast tumors after chemotherapy also displayed typical features of cellular senescence (te Poele et al. 2002).

Depending on the initiating oncogene, transformation relies on fail-safe defects that disrupt either apoptosis or senescence. There are a number of reports that drug-inducible senescence could become detectable only after apoptosis has been disabled (Maloney et al. 1999). It is conceivable that senescence occurs with much slower kinetics, serving as a 'backup' fail-safe program in case the first-line response is corrupted. This is supported by sequential disruption of apoptosis- and senescence-controlling genes during tumor formation and subsequent therapy reported in human cancers (Carter et al. 2001; Elenitoba-Johnson et al. 1998).

7.4 Senescence Based Therapy

Different chemical agents can induce cellular senescence epigenetically. Treatment of primary cells with H_2O_2 or butyrate provokes early senescence (Chen 2000). Similar results were obtained after treatment with high doses of radiation and other damaging agents (Chen 2000). Interestingly, the treatment of different tumor cell lines with different chemotherapeutic agents, radiation or differentiating agents induces irreversible growth arrest, with enzymatic and morphologic changes resembling those occurring during replicative senescence. Moderate doses of doxorubicine induced a senescent phenotype in 11 out of 14 tumor cell lines analysed, independently of p53 status (Chang et al. 1999a). A similar effect has been observed in lines from human tumors treated with cisplatin (Wang et al. 1998b), hydroxyurea (Yeo et al. 2000) and bromodeoxyuridine (Michishita et al. 1999). In mammary carcinoma cell lines treated in vitro and in vivo with differentiating agents, terminal proliferative arrest with minimal toxicity for normal cells has been observed (Chang et al. 1999b).

The propensity of tumor cells to undergo senescence in response to different kinds of damage induced by commonly used chemotherapeutic treatments was compared on cell lines from different tumor origins Elenitoba-Johnson et al. 1998. Under equitoxic doses, the strongest induction of a senescent phenotype was observed with DNA-interacting agents (doxorubicin, aphidicolin and cisplatin) and the weakest effect was observed with microtubule-targeting drugs (Taxol and vincristine). A medium response was observed with ionising radiation, cytarabine and etoposide. Induction of senescence by the drugs was dose dependent and correlated with the growth arrest observed in the cultures (Chang et al. 1999b; Chen 2000; Michishita et al. 1999; Yeo et al. 2000). The drug-induced senescent phenotype in tumor cells was not associated with telomere shortening and was not prevented by the expression of telomerase (Elmore et al. 2002).

Drug-induced senescent phenotypes have been confirmed in vivo (Schmitt et al. 2002). A study from Poele et al. (2002) revealed the correlation between chemotherapeutic treatment in clinical cancer and the senescence response. In

frozen samples from breast tumors treated by neoadjuvant chemotherapy (cyclophosphamide, doxorubicin and 5-fluoracyl), senescent markers were detected in 41 % of samples from treated tumors. Normal tissue was negative, suggesting that the chemotherapy-induced senescence was a specific response of tumor cells. Interestingly, senescence response was associated with wild type p53 and the increased expression of p16. Similarly, in treatment-induced senescence, murine Eμ-myc lymphoma response required wild type p53 and p16 (Schmitt et al. 2002). It is interesting to explore whether the efficacy of current therapeutic regimes applied to tumors depend on the de novo senescence-induced phenotype, and whether there is any difference according to the tissue of origin, i.e. Mesenchymal vs epithelial.

The Chk2 kinase is a tumor suppressor and key component of the DNA damage checkpoint response that encompasses cell cycle arrest, apoptosis, and DNA repair. It has also been shown to have a role in replicative senescence resulting from dysfunctional telomeres. Some of these functions are at least partially exerted through activation of the p53 transcription factor. High-level expression of Chk2 in cells with wild type p53 led to arrested proliferation with senescent features (Gire et al. 2004). These were accompanied by p21 induction, consistent with p53 activation. However, Chk2-dependent senescence and p21 transcriptional induction also occurred in p53-defective cells. Small interfering RNA-mediated knockdown of p21 in p53-defective cells expressing Chk2 resulted in a decrease in senescent cells. DNA-damage response is also induced by cytokines, such as interferons. Sustained treatment with interferon triggers a p53-dependent senescence program. Interferon-treated cells accumulated gamma-H2AX foci and phosphorylated forms of ATM and CHK2. The DNA damage signalling pathway was activated by an increase in reactive oxygen species (ROS) induced by interferon and was inhibited by the antioxidant N-acetyl cysteine. RNA interference against ATM inhibited p53 activity and senescence in response to beta-interferon (Moiseeva et al. 2006). It seems that p53 activation is the primary response to DNA damage, but its absence does not preclude a response with a senescent phenotype.

Comparable to p53, which functions as a fail-safe mediator of DNA-damage response, the p16 inhibitor has been implicated in both response to DNA-damage and control of stress-induced senescence. Although the molecular mechanism used by p16 to control not only temporary but permanent cell cycle arrest is unclear, p16 responds to DNA-damage in a delayed manner and appears to be indispensable for the maintenance of cellular senescence (Schmitt et al. 2002; te Poele et al. 2002). A synthetic inhibitor of CDK4/CDK6, possibly mimicking the role of p16, produced a DNA-damage-independent form of senescence in cells lacking p16 expression and inhibited the growth of tumors in mice (Roberts et al. 2012). This inhibitors is being shown to produce great effect in human clinical trials (Flaherty et al. 2012), but at present we do not know if this efficacy is caused by the CDK4/cell cycle inhibition or by the senescence induction.

Use of siRNAs to inactivate the papilomavirus oncoproteins E6 and E7, which deregulate p53 and pRb, restored cellular senescence in cervical cancer cells. Introduction of E2 protein, a negative regulator of E6 and E7, induced senescence

in almost all cervical carcinoma cells tested. The effect of E2 was not accompanied by telomere shortening, nor was it prevented by telomerase expression. Induction of senescence by E2 was associated with p53 stabilisation and strong induction of p21, and it was prevented by using p21 antisenses (Wells et al. 2000).

Many observations indicate that p53, p21 and p16, which regulate cellular senescence, play an important role in treatment-induced senescence of tumor cells. Since these genes are commonly lost in human tumors, we can expect that most human tumors do not respond by undergoing senescence. However, this is not the case. Chemotherapeutic drugs induced senescence in p53- and p16-defective tumor cell lines (Chang et al. 1999b). In vivo, 20 % of tumors undergoing senescence after treatment showed p53 mutations (te Poele et al. 2002). We have been able to induce senescence with several chemotherapeutic drugs in p53-null cells independently of p16 (Moneo and Carnero, unpublished). We have found that the induced senescence correlated with p53-independent p21 induction. Moreover, knock-out of p53 or p21 in HCT116 cells decreased but did not abolish cellular senescence. Hence, p16, p53 and p21 might acts as positive regulators but are not absolutely required for this response. Other related tumor suppressors, such as p63 or p73, could be involved, and their role in drug-induced senescence should be explored.

Treatment with 6-anilino-5,8-quinoline quinone, a previously described inhibitor of guanylate cyclase, induced cellular senescence (Lodygin et al. 2002). Microarray analysis revealed that this compound induced the Cdk inhibitor p21WAF1 in a p53-independent manner. Furthermore, p21, though not p53, was required for inhibition of proliferation by the drug. The lack of p53 involvement suggests that this compound acts independently of DNA damage induction. Growth inhibition was also observed in malignant melanoma and breast cancer cell lines. Functional inactivation of the retinoblastoma tumor-suppressor protein converted 6-anilino- 5,8quinolinequinone-induced growth arrest into apoptosis. Tumor cell senescence was also found to be induced by TGFb and by differentiating agents including retinoids. The induction of senescence has been analyzed in more detail with derivatives of vitamin A, which regulate cell growth and differentiation through their effects on gene expression (Roninson and Dokmanovic 2003).

7.4.1 Telomerase Inhibitors

Restoration of the limited replicative potential in tumors as an anticancer therapy has been widely examined through the targeting of telomerase activity. Early studies indicated that telomerase activity is absent in somatic tissues and present in most cancers (Kim et al. 1994). It was therefore reasonable to suggest that inhibition of telomerase activity, with a consequent shortening of telomeres and arrest of cell growth, might be an effective treatment of cancer.

Several different approaches to telomerase inhibition have been adopted to prevent the multiplication of neoplastic cells in culture. These have included treatment of the cells with the alkaloid berberine, transfection with an antisense vector for the

human telomerase RNA component, introduction of a catalytically inactive, dominant-negative mutant of human telomerase reverse transcriptase and low-level expression of a mutant-template telomerase RNA. All of the treatments inhibit the multiplication of neoplastic cells in culture, and those tested also inhibit tumor formation in mice. It should however be noted that the transfection of neoplastic cells with telomerase-inhibitory vectors was accomplished either in culture before their inoculation into mice or (in the case of the antisense RNA) through daily injections into the growing tumors for 7–14 days. No attempt was made to assess the long-term systemic injection of vectors into mice carrying the tumors, leaving the matter of effects on normal cell function yet to be investigated. Telomere shortening has been observed in the treated tumor cells and correlates with inhibition of their proliferation (Hahn et al. 1999). The expression of threshold levels of mutant-template telomerase RNA decreases cell viability despite the retention of endogenous wild- type telomerase RNA, wild-type telomerase activity, and unaltered stable telomere lengths.

One reported advantage of telomerase inhibition as a cancer chemotherapy was that it was not expected to induce cancer in normal cells, as telomerase activity is closely associated with advanced tumors (Kim et al. 1994). Knockout of the gene for the RNA component of telomerase in mice does not, however, prevent either tumor formation or neoplastic transformation of cells cultured from such mice (Blasco et al. 1997; Rudolph et al. 1999). The incidence of spontaneous malignancies is even higher than that of normal mice (Rudolph et al. 1999). A similarly increased risk of cancer is found in individuals with the inherited syndrome dyskeratosis congenita (DKC) that is caused by a mutation in one of the components of telomerase, such that individuals with DKC are deficient for telomerase activity (Vulliamy et al. 2006). This increased incidence of cancer is presumably a result of end-to-end fusion of chromosomes destabilized by inadequate capping (Marciniak and Guarente 2001). There is therefore the distinct possibility that systemically introduced inhibition of telomerase in cancer chemotherapy would increase the frequency of chromosome aberration and the risk of secondary cancers in normal tissue, particularly when p53 mutations already exist (Artandi and DePinho 2000).

The situation became more complicated when it was found that telomerase activity is present in stem cells and dividing transit cells of renewing tissues, and even when cell division is induced in tissues conventionally regarded as quiescent. Thus, it seems likely that all tissues with cells able to divide have either ongoing or potential telomerase activity with a capacity for telomere maintenance during cell division.

Treatment of cancer by telomerase inhibition is still considered potentially valid for several reasons that might mitigate side effects on normal tissues (Holt et al. 1997). One reason is that telomeres are longer in normal tissues than in most cancers, and treatment of tumors can be designed to end before telomere depletion in normal tissues (Artandi and DePinho 2000). However, further studies with this approach must be carried out to protect renewing tissues, such as intestine, epidermis, and hematopoietic tissue, in which stem cells and transit cells are constantly dividing at a high rate.

It is expected that telomerase inhibitors will be developed that have far fewer side effects than many of the cancer chemotherapeutic agents that are currently available. Individuals with DKC show features that include abnormalities of the skin and nails and eventual failure of proliferation in the bone marrow, which indicates that telomerase is required for normal proliferative capacity in these somatic tissues. Despite this telomerase deficiency, onset of pancytopaenia in these individuals does not occur until a median age of 10 years, which indicates that it might be relatively safe to administer telomerase inhibitors continuously for several years.

Telomerase inhibitors will not be useful, however, for the minority of tumors that use ALT. In addition, in telomerase-positive tumors it can be predicted that effective telomerase inhibitors will exert an extremely strong selection pressure for the emergence of resistant cells that use the ALT mechanism. Activation of ALT was not observed in cell-culture experiments in which telomerase-positive cell lines were treated with small-molecule inhibitors of telomerase or dominant-negative TERT mutants (Zhang et al. 1999), indicating that it is not a high-frequency event. This might be a problem, however, in clinically significant tumors containing as many as 10^{12} cells. Development of ALT inhibitors may therefore be necessary. For tumors that use both telomere maintenance mechanisms, treatment might need to be initiated with a combination of telomerase and ALT inhibitors. Both telomerase and ALT must access the telomere, but how this might be achieved is at present unknown. A further possibility could be to identify molecular targets for simultaneous inhibition of both telomere maintenance mechanisms since proteins involved in telomerase-based and ALT-mediated events may overlap.

7.5 Concluding Remarks

The concept of senescence as a barrier to tumorigenesis, either by natural replicative limits or as stress-induced senescence leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis and therapy. It is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation and differentiating agents. These senescent features can be imposed even in the absence of the two functional effector pathways, p53 and pRb. This lead to speculate the possible benefits of inducing an unspecific senescence program to stop tumor growth. This might be of value added to surgery or radiation, however, possible escape from a yet uncontrolled senescent phenotype and the unknown effect in vivo of senescent stromal cells might hamper these efforts. A more controlled induction of senescence through the knowledge of pathways involved and targeting specific targets might rend a less profitable but more valued effort. The use of tools such as oncolytic viruses driven by telomerase promoters might also work better than direct inhibition of the protein. However, it is too early and more research is needed in the basic understanding of the molecular mechanisms driving the senescence processes before embarking patients in such therapy.

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Chapter 8
Pro-senescence Therapy for Cancer: Time for the Clinic

Madhuri Kalathur, Diletta Di Mitri, and Andrea Alimonti

Abstract Cellular senescence is a stable cell cycle arrest that occurs in diploid cells during aging. However, diploid cells can also experience an accelerated senescence response, termed pre-mature senescence, driven by DNA-damage, oncogene over-expression or loss of tumour suppressor genes. Recent evidences demonstrate that cellular senescence occurs also in tumours, where it opposes tumour initiation and progression. Senescence cells secrete a variety of cytokines and secreted factors, known as the senescence secretory phenotype (SASP), that regulate both the senescence entry and maintenance and can propagate senescence to adjacent cells, acting in a cell non-autonomous manner. In addition, SASP can also promote the recruitment and activation of immune cells that in turn mediate the clearance of senescent cancer cells.

Several novel strategies have been developed to promote senescence in tumours. This approach has been named pro-senescence therapy for cancer. Among the most promising pro-senescence therapies for cancer there are compounds that can enhance or reactivate p53 in tumours, that blocks the cell cycle machinery and inhibit telomerase. These compounds are currently in both pre-clinical and clinical trials. In addition, SASP reprogramming or induction of PTEN-induced cellular senescence have recently been proposed as a promising therapeutic approach for the treatment of certain types of tumours. In conclusion, many essential proves of the biological relevance of senescence in cancer have been currently discovered and strategies aimed to identify novel pro-senescence compounds would aid the development of more efficient treatment modalities for cancer therapy.

Keywords Cellular senescence • Senescence-associated secretory phenotype • Tumor suppressor • Oncogene • Oncogene-induced senescence • RAS • BRAF • PTEN • PICS • p53 • Immune response • Pro-senescence therapy • Cancer therapy

M. Kalathur • D. Di Mitri • A. Alimonti (✉)
Institute of Oncology Research (IOR), Oncology Institute of Southern Switzerland (IOSI),
Via Vincenzo Vela 6, CH6500 Bellinzona, Switzerland
e-mail: andrea.alimonti@ior.iosi.ch

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8.1 Cellular Senescence

Cellular senescence is a stable cell cycle arrest that occurs in diploid cells during aging. The first description of cellular senescence dates back to 1965 when Hayflick and Moorhead observed that primary human fibroblasts failed to replicate after about 50 cell divisions (Hayflick and Moorhead 1961). This biological clock was called "Hayflick limit". Later research demonstrated that the progressive shortening of telomeres during human cellular replication are at the basis of the Hayflick limit and that cell cease to grow accumulating DNA damage (Xu et al. 2013). This mechanism also appears to prevent genomic instability. Since this type of cellular senescence depends on cellular replication, it was also termed replicative senescence. However diploid cells can also experience an accelerated senescence response termed pre-mature senescence (Serrano et al. 1997). Initially thought to be an artifact induced by cell culture stress, premature cellular senescence is now a well-established process that can be triggered through either the activation of oncogenes (a type of senescence that is termed oncogene-induced senescence (OIS) or loss of tumour suppressor genes. Recent studies demonstrate that this type of cellular senescence response represents a complex biological process, occurring also in vivo (Counter et al. 1992; Blasco et al. 1997; Ben-Porath and Weinberg 2004; Nardella et al. 2011). Pre-mature senescence can be induced by genotoxic stress including oxidative stress and ionizing radiation (Sherr and DePinho 2000). In addition to this, treatments with X Rays or chemotherapeutic drugs can induce premature senescence in cancer cells (Dorr et al. 2013), a mechanism known as treatment induced senescence (TIS). Finally, it has been recently demonstrated that senescence can occur in vivo in different tumor models, where it arrests tumor development and progression. This finding represents an essential prove of the biological relevance of senescence in cancer, and it has paved the way for treatments that enhance this process for cancer therapy (Fig. 8.1).

8.1.1 Hallmarks of Cellular Senescence

Senescent cells are not characterized by a specific biomarker. Expression of a group of markers defines a senescent cell, however senescent cells not always express all the markers. Cell cycle growth arrest is a crucial mechanism for the identification of all types of senescence, both in vitro and in vivo. Nevertheless, as multiple cellular mechanisms can drive a stable replicative arrest in cells, cell cycle exit cannot be considered a unique marker for senescence. Senescent cells are commonly characterized by an increased cell size and a more flattened shape than normal cells and frequently exhibit an increased expression of senescence associated β-galactosidase activity (SA-β-gal) that in these cells can be detected at pH 6. Also augmented levels of p19ARF, p53, PAI-1 and of the cell cycle inhibitors p16INK4a, p21/CIP1 and p27 are generally ascribed as senescence biomarkers. In addition to this, senescence cells may exhibit senescence associated heterochromatin foci formation (SAHF), persistent DNA damage response (DDR) and commonly secrete large number of growth factors, cytokines

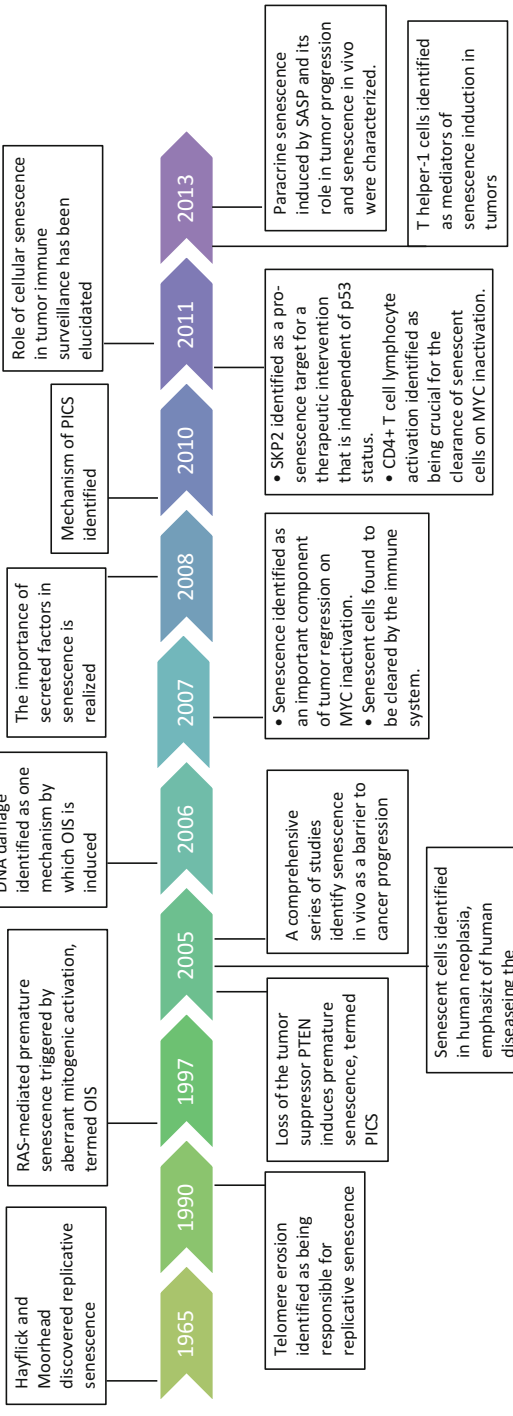

Fig. 8.1 Milestones in senescence research: a timeline

and proteases, known as the senescence associated secretory phenotype (SASP). All the above-mentioned factors together define the hallmarks of senescence (Collado et al. 2007; Kuilman et al. 2010). Nevertheless, some of the cited senescence markers still need to be confirmed in vivo, and it is still necessary to clarify to what extend they can be considered valid across tissues and species.

8.1.2 Causes of Cellular Senescence

8.1.2.1 Telomere Shortening

Telomeres are regions of repetitive nucleotide sequences that protect the end of the chromosome from deterioration or from fusion with neighboring chromosomes. Telomeres shorten with each cell division (S phase) and each round of DNA replication leaves 50–200 bp of unreplicated DNA at the 3′ end. Telomerase adds bases to the ends of telomeres maintaining the function of these repeats. As cells divide repeatedly, there is not enough telomerase to compensate telomeres erosion, so the telomeres grow shorter and the cells age. Cancer cells escape senescence by activating telomerase enzyme and by other mechanisms, which prevent the telomeres from getting shorter (Shay 2014), thus allowing them to proliferate indefinitely. This makes telomerase an attractive target for the development of novel anti-cancer therapeutic agents (Wong et al. 2013). Telomeres are subject to attrition due to the fact that DNA polymerase fails to completely replicate the lagging strands. In the early 1970s, Olovnikov (1971) and Watson (1972) independently described this so-called "end replication problem", which contributes to telomere shortening. Thus, telomeres act as a molecular clock, reflecting the replicative history of a primary cell (Harley 1990). When telomeres reach a critical minimal length, their protective structure is disrupted. This triggers a DNA damage response (DDR), which is associated with the appearance of foci that stain positive for γ-H2AX (a phosphorylated form of the histone variant H2AX) and the DDR proteins 53BP1, NBS1, and MDC1. Moreover, the DNA damage kinases ATM and ATR are activated in senescent cells (d'Adda di Fagagna et al. 2003). After amplification of the DDR signal, these kinases activate CHK1 and CHK2 kinases. Communication between DDR-associated factors and the cell cycle machinery is brought about by phosphorylation and activation of several cell cycle proteins, including CDC25 (a family of phosphatases) and p53. In addition to this, differential expression of p53 isoforms has been linked to replicative senescence (Fujita et al. 2009). Together, these changes can induce a transient proliferation arrest, allowing cells to repair their damages. However, telomere shortening does not completely explain the reason why mammalian cells age. Some mammals such as mice have longer telomeres that human telomeres and express higher telomerase levels (Kipling and Cooke 1990; Prowse and Greider 1995). However mice life span is significantly shorter that human life span. Elongation of cells life span can be achieved by culturing human cells in serum-free medium supplemented with a number of defined

growth factors (Loo et al. 1987) or by culturing cells under physiological oxygen conditions (Parrinello et al. 2003). In contrast, oxidative stress induces senescence in cultured human cells (Packer and Fuehr 1977; Chen et al. 1995; Yuan et al. 1995). Thus, the immortalization of mammalian cells requires not only telomere maintenance, but also optimal culture conditions (Mathon et al. 2001; Ramirez et al. 2001; Tang et al. 2001; Herbert et al. 2002) (for review, see Wright and Shay (2002)). Oxidative stress-mediated DNA damage is also an important determinant of telomere shortening (Richter and von Zglinicki 2007). Zhou et al. (2014) have demonstrated that ER-stress transiently activates the catalytic components of telomerase (TERT), depletion of hTERT sensitizes cells to undergo apoptosis, whereas increased hTERT expression reduces ER stress-induced cell death independent of catalytically active enzyme or DNA damage signaling. Recently, it was also demonstrated that lifestyle modification like diet, activity, stress management, and social support have resulted in increased telomere length and telomerase activity (Ornish et al. 2013) (Fig. 8.2).

8.1.2.2 Stress-induced Cellular Senescence

Contrary to replicative senescence, stress induced senescence does not depend on telomere length. Extrinsic and intrinsic physiological stresses are reported to promote senescence in normal and cancer cells. Extrinsic stresses include treatment with DNA damage agents such as hydrogen peroxide (Chen and Ames 1994), UV- or γ- irradiation (Rodemann et al. 1989), tert-butylhydroperoxide (Toussaint et al. 1992) or different anti-cancer chemotherapy drugs (Sanchez-Prieto et al. 2000; Benhar et al. 2002). Intrinsic stresses include subjecting cells to abnormal O_2 levels (von Zglinicki et al. 1995) and inadequate nutrients and culturing condition. This last phenomenon is also termed "culture shock" (Sherr and DePinho 2000), applies to both human and mouse cells and it is independent from telomere length. Oxidative stress, ER-stress and interferon-related responses, and signaling via either insulin growth factors (IGF) or mitogen activated protein kinases (MAPK), are various stress pathways that also cause cellular senescence (Campisi and d'Adda di Fagagna 2007). These stresses initiate various cellular signaling pathways, which turn to activate the p53 protein, the Rb protein, or both pathways. Distinguishing between replicative senescence and stress-induced senescence is very subtle, as these titles merely reflect the fact that a spectrum of different stimuli feed into one response program or other.

8.1.2.3 Overexpression of Oncogenes

Several oncogenes induce senescence both in vitro and in vivo. Oncogene induced senescence (OIS) is regulated by a complex signaling network that opposes the process of malignant tumor formation from benign tumors. OIS driven by HRASG12V or BRAF overexpression in human fibroblasts is triggered by massive

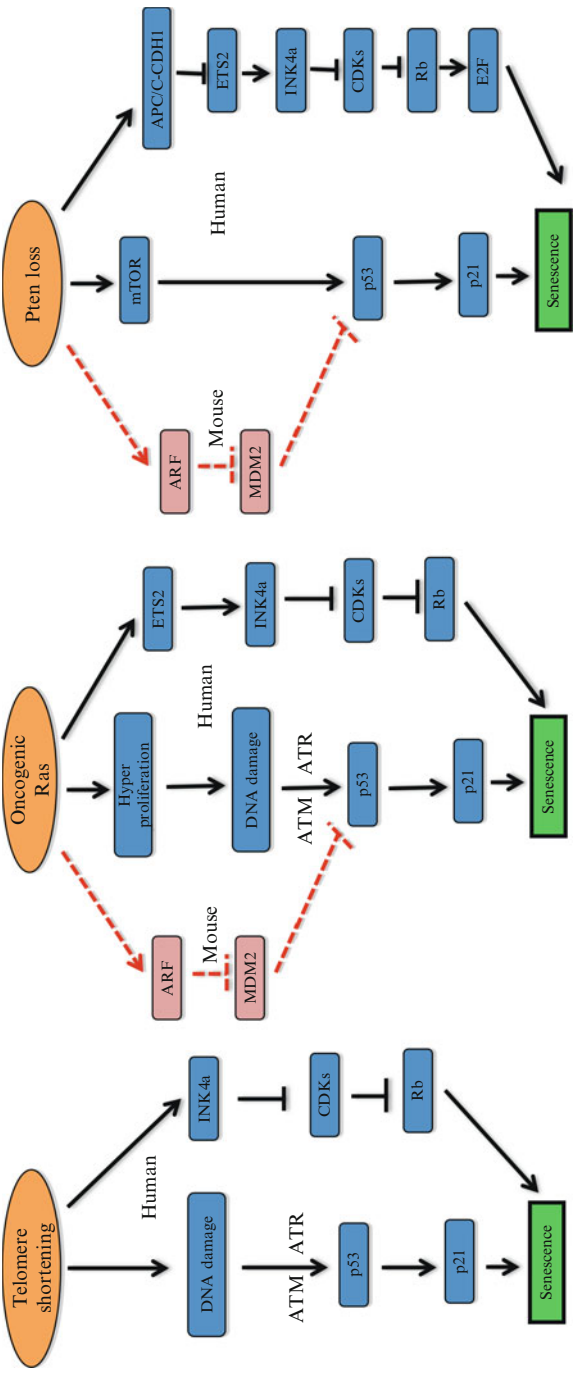

Fig. 8.2 Causes of cellular senescence. Telomere shortening, overexpression of specific oncogenes (e.g. Ras), and loss of tumor suppressor genes (e.g. Pten) as major mechanisms underlying cellular senescence

hyperproliferation and DNA hyper-replication. This in turn sparks the activation of an S phase specific DDR that promote senescence (Di Micco et al. 2006). Although this DDR is initiated by a mechanism that is distinct from that of replicative senescence, it shares with the latter effectors and primary pathways and also results in the formation of senescence-associated DNA damage foci (SDF formation) (d'Adda di Fagagna 2008). Hyperproliferation and DDR are essential for OIS. Indeed OIS cannot be triggered in cells that lack DDR such as ATM/ATR deficient cells or in cells pre-treated with aphidicolin, a selective cell cycle inhibitor (Di Micco et al. 2006) (Fig. 8.2).

RAS

RAS was first identified in 1964 as a human oncogene and was described as a transforming retrovirus that produces tumors in mice. Mutations in oncogenic HRAS are most common in a wide variety of human cancers and are found in 30 % of the tumors (DeNicola et al. 2011). At least five inherited mutations in the HRAS gene have been identified in people with Cosetello syndrome. Each of these mutations changes a single amino acid in a critical region of the HRAS protein. The most common mutation replaces the amino acid glycine with the amino acid serine at position 12. Ras requires cooperation of another oncogene or inactivation of tumor suppressor genes like p53 and p16INK4a for transformation. Over expression of oncogenic HRAS in primary cells results in a permanent form of cell cycle arrest called oncogene induced senescence. Ras-induced senescence is accompanied by the upregulation of the tumor suppressors PML, p53 and p16INK4a (Serrano et al. 1997). Oncogene-induced senescence is executed via p53 and p16INK4a, which are involved in proliferation, differentiation and cell death. Inactivation of p53 and p16INK4a proteins results in bypassing Ras-induced senescence. This explains why Ras-induced senescence evolved as a protective barrier to prevent tumor progression (Courtois-Cox et al. 2008). p53 is an essential regulator of OIS (Ferbeyre et al. 2000; Wei et al. 2001), through transcriptional activation of target genes, in particular *CDKN1A* (encoding the protein p21/CIP1) (Serrano et al. 1997). In OIS, the activation of p53 is driven by phosphorylation (Di Micco et al. 2006), with concomitant stabilization of the protein mediated by ARF induction (Serrano et al. 1997). However, ARF induction may have a more limited role for the upregulation of p53 in a human context (Brookes et al. 2002). In addition to p53, OIS also engages other senescence effectors, including p16INK4a (for example, through ETS2 (Ohtani et al. 2001) and derepression of the genomic locus through inactivation of the polycomb group complex (Bracken et al. 2007). Moreover there are now several mouse models providing physiological evidence for OIS in vivo (Braig et al. 2005; Collado et al. 2005; Dankort et al. 2007; Sarkisian et al. 2007). Interestingly, the senescence response that is induced through the loss of the *Rb1* tumour suppressor gene in vivo has also been reported to display OIS-like features that are mediated by activation of NRAS (Shamma et al. 2009). Transcriptional repression of pro-proliferative genes, like E2F target genes, and the senescence associated

secretory phenotype (IL-6, IL-8, PAI-1) can also induce and maintain senescence driven by RAS over-expression (Dimauro and David 2010).

BRAF

BRAF belongs to the serine threonine protein kinase family and acts downstream of oncogenic RAS by activating the MEK-ERK cascade in tumors. BRAF is well studied in the context of melanoma as 70 % of the cases carry activated BRAF mutations. BRAF can be activated by a single amino acid substitution. The glutamic acid to valine substitution (BRAFV600E) is the most commonly occurring, as it is present in 90 % of the cancers involving this gene (Dhomen and Marais 2007). Study of BRAF mutations in Nevi is an interesting model of oncogene-induced senescence. Nevi are benign tumors of melanocytes in which BRAF mutations occur at a very high frequency. Activated BRAF favors the establishment of senescence, rather than its progression towards melanoma. Recent studies demonstrate that BRAFV600 over-expression in melanocytes contributes to an initial proliferative burst of melanocytes followed by a growth inhibitory response associated with a stable proliferation arrest, an increase in p16INK4a and expression of senescence associated SA-β-gal activity (Michaloglou et al. 2005). As shown in RAS, BRAF needs to cooperate with other activated oncogenes or loss of tumor suppressors to escape senescence effect. A recent paper demonstrates that BRAF-induced senescence depends on the secreted protein IGFBP7 and that loss of this protein is a critical step for the development of melanoma (Wajapeyee et al. 2008). BRAFV600E also cooperates with loss of Pten to promote tumor progression and metastatic melanoma (Dankort et al. 2009).

8.1.2.4 Loss of Tumor Suppressor Genes and Senescence

Similar to over expression of oncogenes, tumor suppressor genes loss can also trigger senescence in vitro and in vivo. Senescence induced by loss of tumor suppressor genes (TSG) was first reported for the tumor suppressors PTEN and NF-1. Complete loss of PTEN induces a senescence response termed PTEN loss induced cellular senescence (PICS) that is triggered by p53 activation (Alimonti et al., 2010 b). Both mTOR-induced p53 translation and ARF mediated p53 stabilization cooperate to establish PICS. *Pten* loss in the mouse prostate promotes the formation of a benign prostate tumor lesion. However loss of p53 in *Pten*$^{-/-}$ cells and mouse prostate epithelium by-passes PICS, triggering prostate tumor invasiveness (Chen et al. 2005). Loss–of-function or mutations in NF-1, the gene involved in type-I neurofibromatosis, is also associated to senescence both in vitro and in vivo. Neurofibromatosis is a tumor disorder characterized by the development of benign tumor lesions along the nervous system. Histological analyses have demonstrated that dermal neurofibromas are SA-β-gal and p16INK4a positive (Courtois-Cox et al. 2006). NF-1 converts RAS to its inactive GDP form, and reduced levels of NF-1 expression inhibit

RAS activity by shutting down its downstream signaling resulting in profound senescence effects (Bardeesy and Sharpless 2006; Courtois-Cox et al. 2006). *VHL* (von Hippel-Lindau tumor suppressor gene) is the most commonly mutated TSG in renal cell carcinomas and haemangioblastomas in humans. Acute inactivation of *VHL* in the mouse causes the formation of benign renal tumor lesions characterized by a senescence phenotype. VHL loss induces senescence by up regulating both Rb and p27 in a p53 and Hif-dependent manner. VHL-associated neoplasias need to overcome loss of senescence induced by VHL to become aggressive (Young et al. 2008). RB1 loss in thyroid cells induces the formation of benign adenomas characterized by increased levels of senescence markers. RB1 loss promotes elevated NRAS activity that in turn induces a DNA damage response and p130-dependent cellular senescence (Shamma et al. 2009). This effect is mediated by E2F activation. On this line, a transgenic mouse model over-expressing E2F3 protein in the intermediate lobe of the pituitary gland developed hyperplasia and senescence in the mouse pituitary gland (Lazzerini et al. 2006) (Fig. 8.2).

8.1.3 Autocrine and Paracrine Senescence

Recent findings demonstrate that senescent tumor cells secrete a variety of immune modulators and inflammatory cytokines, referred to as the senescence-associated secretory phenotype (SASP) or senescence secretome, that mediates contradictory effects. The SASP can stimulate the innate and adaptive anti-tumor immune response (a process designated as "senescence surveillance") leading to tumor clearance (Xue et al. 2007; Kang et al. 2011) but can also promote tumorigenesis by supporting the proliferation of neighboring tumor cells (Rodier et al. 2007; Coppe et al. 2010; Davalos et al. 2010) or by hindering chemotherapy efficacy (Jackson et al. 2012). Oncogene induced senescence is tightly regulated by the SASP. Recent evidences in OIS demonstrate that senescence entry and maintenance depends on the functional integrity of the chemokine receptor 2 (CXCR2), receptor of interleukine 8 (IL8). Interestingly, inactivation of this receptor blocks both replicative senescence and OIS. Indeed cells undergoing OIS secrete multiple CXCR2-binding chemokines that acts in an autocrine manner to reinforce senescence. The release of these chemokines is regulated by NF-kappaB and C/EBPβ transcription factors and coordinately induces CXCR2 expression (Acosta et al. 2008). Another paper demonstrated that IL6 is also required for the execution of OIS, in a cell-autonomous mode. IL6 depletion caused the SASP to collapse and abolished senescence entry and maintenance (Kuilman et al. 2008). Interestingly the transcription factor C/EBPβ cooperates with IL6 to amplify the activation of the inflammatory network, including IL8.

Interestingly, senescent cells can also induce senescence in normal cells acting in a paracrine manner, through the secretion of cytokines and secreted factors in the tumor microenvironment. Therefore senescence can be propagated in tumors and normal tissues in a non cell autonomous manner. Among other factors that can

induce paracrine senescence, TGF-β family ligands, VEGF, CCL2 and CCL20 were identified as major regulators. Importantly, IL1α can control paracrine senescence by modulating the SASP of OIS. Indeed IL1α expression can reproduce SASP activation in normal cells, resulting in senescence (Acosta et al. 2013). Therefore, paracrine senescence acts by reinforcing the arrest of cells undergoing OIS. Inactivation of IGFB7, a gene that encodes for a secreted protein, can also control BRAFV600E induced cellular senescence acting in a paracrine manner. Interestingly IGFB7 blocks the activation of the MAPK induced by BRAFV600E over-expression, promoting cell cycle arrest. Therefore IGFB7 acts as a paracrine factor that can reinforce senescence after the initial proliferative outburst induced by BRAF (Wajapeyee et al. 2008).

8.1.4 Autophagy and Senescence

Autophagy is a genetically regulated program responsible for balancing sources of energy in many physiological conditions. This process plays an essential role in removing misfolded or aggregated proteins, clearing damaged organelles, and eliminating intracellular pathogens. Autophagy is generally considered to be a survival mechanism, nevertheless cellular stress can also activate this process, and recent findings suggest that autophagy may be considered an important tumor suppression mechanism. Young and colleagues lately depicted a new role for autophagy as an effector mechanism of senescence. Indeed, autophagy was upregulated upon OIS induction in human fibroblasts, as indicated by accumulation of autophagosomes and increased protein degradation, and was shown to mediate senescence establishment in vitro. These results were further confirmed in murine papillomas in vivo, suggesting that autophagy may play a critical part in tumor-suppression by supporting the role of senescence as a barrier against tumorigenesis (Young et al. 2009). Interestingly, Dorr and colleagues recently showed that therapy-induced senescence mediates a metabolic reprogramming of cancer cells and a consequent strong proteotoxic stress. Interestingly, cancer senescent cells rely on autophagy as a source of energy to counteract this cellular stress and to fuel the senescence response. Such liability of tumor cells on autophagy may be used to selectively eliminate senescent cancer cells in vivo, thus rendering autophagy therapeutically targetable in tumors (Dorr et al. 2013).

8.1.5 Oncogene Induced Senescence and Immune Response

The role of cellular senescence as a barrier to tumorigenesis both in vitro and in vivo has been deeply investigated. Nevertheless, recent findings indicate that tumour regression associated with senescence response also involves non cell-autonomous processes. Xue and colleagues recently showed that p53 reactivation in a liver

cancer mouse model expressing oncogenic Nras ($Nras^{G12V}$), leads to a strong tumour involution associated with senescence induction and with an inflammatory reaction that involves cell subsets from the innate immune response, such as neutrophils, macrophages and natural killer cells. In this model, p53 activation and senescence provoke an up-regulation of cytokines known to recruit and activate cells from the immune response (Xue et al. 2007). These evidences were further supported by a follow up manuscript from Zender and colleagues, indicating that antigen-specific $CD4^+$ T lymphocytes infiltrating the tumour indirectly mediate the clearance of senescent cells in liver cancer by orchestrating monocytes and macrophages activation (Kang et al. 2011). All together these findings support a model made of a cascade of events, designated as "senescence surveillance", in which cell senescence triggers the recruitment of immune cell subsets and consequently primes an inflammatory response that flows into the clearance of senescent tumours cells. Importantly, the senescence surveillance cannot be ascribed to a single immune cell subset, but it seems to rather depend on the cooperation of distinct cell types (Xue et al. 2007; Kang et al. 2011; Iannello et al. 2013). Multiple findings indicate that cell senescence may also be induced and regulated by non cell-autonomous mechanisms. In line with this evidences, Braumuller and colleagues recently showed that tumour-infiltrating T-helper 1 lymphocytes can trigger senescence in p53 null pancreatic tumors (RIP-Tag2 mouse model). Senescence induction in this model was antigen restricted and was mediated by IFN-γ and TNF-α release from Tag-specific T cells. Interestingly, the permanent growth arrest of β-cells was strictly depending on TNFR1 signaling, and was fully abrogated in absence of p16INKa and p19ARF expression. Notably, IFN-γ and TNFα was not restricted to Tag-expressing β-cancers as this mechanism of action provoke senescence induction in several mouse and human cell lines in vitro (Braumuller et al. 2013). All together these findings add new insights on the interaction existing between tumor-infiltrating immune cell subsets and senescent cells in cancer and may flow into new immunotherapic strategies for the treatment of human cancer.

8.2 PTEN-Loss Induced Cellular Senescence

8.2.1 PTEN Function

PTEN was originally discovered as the tumor suppressor gene frequently lost on chromosome 10q23 (Li et al. 1997). Subsequent studies demonstrated that loss of PTEN function resulted from several genetic mechanisms including small-scale PTEN gene mutations (point mutations, insertions, small deletions), allelic loss at chromosome 10 and epigenetic silencing via hyper-methylation of the PTEN promoter region (Salmena et al. 2008). The relevance of PTEN in cancer has been addressed through the generation of germline knockout *Pten* mice by several independent laboratories (Di Cristofano et al. 1998; Suzuki et al. 1998). These studies

revealed the requirement of Pten for embryonic development. Importantly, heterozygous loss of this tumor suppressor gene in the mouse resulted in the development of cancer of multiple origins as well as in a lethal lymphoproliferative disease (Di Cristofano et al. 1998). In humans, germline loss and mutation of PTEN is observed in a group of autosomal dominant syndromes (PTEN hamartoma tumor syndromes or PHTS) which are characterized by neurological disorders, multiple hamartomas and cancer susceptibility (Hobert and Eng 2009). The most common PHTS associated to PTEN mutations is Cowden Syndrome, followed by Bannayan–Riley–Ruvalcaba syndrome and Lhermitte–Duclos disease. Studies from the mouse have recapitulated a fraction of the features observed in PHTS patients; however, the cooperative genetic or environmental factors contributing to the full symptomatic spectrum in this group of syndromes remain to be defined. PTEN functions as a lipid phosphatase, dephosphorylating the 3′ position of phosphoinoisitde 3,4,5-triphosphate (PIP3). This lipid second messenger is the product of a potent proto-oncogenic kinase, Phosphoinositide 3-Kinase (PI3K), and the trigger for activation of the PI3K pathway (Salmena et al. 2008). The relevance of the PI3K pathway in cancer is highlighted by the elevated number of components within the cascade, whose level or activity is found altered, and represents one of the main targets for cancer therapy (Wong et al. 2010). PTEN can affect tumorigenesis in different modalities. We and others have previously demonstrated that *PTEN* is a bona fide haploinsufficient tumor suppressor gene and that even subtle variations of PTEN levels can initiate tumorigenesis (Alimonti et al. 2010a). These studies demonstrate that PTEN dosage variations rather than PTEN deletions or mutations account for tumor initiation in different models. On this line, recent evidence demonstrate that PTEN expression is regulated by a complex network of miRNAs and that deregulation of this miRNA network affect PTEN levels promoting tumor initiation (Poliseno et al. 2010). Importantly, despite the main role of PTEN as a negative regulator of the PI3K pathway, recent studies report a number of tumor suppressive activities for PTEN that are exerted from within the nucleus, where catalysis of PIP3 does not appear to represent a central function of this enzyme. PTEN phospatase activity can be inhibited by proteins that interact with PTEN such as P-REX2a that are frequently up-regulated in a variety of tumors (Fine et al. 2009). These PTEN interactors can affect the PI3K signaling by increasing the cellular levels of PI3P through inhibition of PTEN in tumors with low frequency of PTEN mutations or deletions. Recent evidences also demonstrate that PTEN has a phosphatase independent function in the nucleus. PTEN mono-ubiquitination mediates its nuclear import and binding to the APC/CDH1 complex arresting cellular proliferation (Trotman et al. 2007). PTEN also binds and stabilizes the centromere in a phosphatase independent manner and PTEN loss drive DNA damage accumulation in immortalized cells (Shen et al. 2007). Finally PTEN complete loss drives a cellular senescence response termed PICS, which depends on the functional activation of mTOR and that can be targeted for cancer therapy (Alimonti et al. 2010b). Taken together all these evidence demonstrate that PTEN affects tumorigenesis at multiple levels and with several modalities.

8.2.2 Loss of PTEN and Senescence

The tumor suppressor activity of Pten leads to a novel paradigm, which is called 'obligate haplo-insufficiency'. Although heterozygous loss of PTEN initiates tumorigenesis, complete loss of Pten activates a p53-dependent cellular senescence response, which acts as a fail-safe mechanism by blocking tumor progression. This senescence response has been named PICS, for PTEN loss induced cellular senescence. As recently shown, PICS depends on the functional activation of p53. Indeed loss of *Pten* doesn't initiate senescence when p53 is mutated, leading to tumor formation and invasiveness (Berger et al. 2011). In characterizing the mechanisms and features of PICS it has been discovered that PICS is a distinct form of cellular senescence with several unique differences from oncogene-induced senescence (OIS) and replicative senescence. Indeed, PICS can occur in arrested cells treated with aphidicolin, a compound which blocks S phase entry and prevents DNA replication. On the contrary, in OIS, aphidicolin treatment results in the abrogation of senescence. Furthermore, pharmacological and genetic inactivation of ATM has no effect on PICS induction. Similar to OIS, p53 has an important role in PICS. However, in this context p53 upregulation is mainly promoted by mTOR-mediated translation[88]. Importantly these findings have been also validated in human cancer cell lines in different studies (Kim et al. 2007). In addition, inactivation of ARF does not dramatically alter p53 levels and senescence in vivo, demonstrating the limited role of ARF for PICS. Finally PTEN loss promotes the upregulation of INK4A through the regulation of ETS2 (Ohtani et al. 2001). Thus, PTEN loss drives senescence in two different manners: through p53 upregulation resulting from mTOR hyperactivation, and through INK4A upregulation resulting from disassembly of the CDH1-containing anaphase-promoting complex (APC/C (also known as the cyclosome)–CDH1) and subsequent accumulation of ETS2. Although it is clear that PICS has a central role in blocking tumour progression in prostate tumorigenesis, the extent to which PICS might restrict the development of other tumors is not clear (Figs. 8.2).

8.2.3 Potential Benefits of PICS

Induction of PICS from a therapeutic prospective offers several advantages. Since PICS does not require hyperproliferation and DDR, treatments that promote PICS in cancer cells will promote a stable cell cycle arrest in absence of genomic instability, avoiding the risk of secondary mutations. Moreover PICS can also occur in non proliferating cells and this property may also be used to induce senescence in quiescent cancer-initiating cells (CICs), that contribute to the maintenance of the tumor in many cancer types. As discussed above, PICS depends on the functional activation of mTOR-mediated p53 translation rather than to ARF mediated p53 stabilization, therefore treatments that stabilize p53 act as pro-senescence compounds in

PTEN null cells. On the contrary, OIS depends on ARF-MDM2 p53 stabilization to be executed (Palmero et al. 1998). As a consequence, in contrast to OIS, the treatment of Pten null cells with MDM2 inhibitors can greatly increase p53 levels. Thus, PICS represents a promising therapeutic option. Moreover it has been demonstrated that PICS can be evoked even in PTEN null tumors that have lost p53. Indeed a novel SKP2 inhibitor an skp2 inhibitor can efficiently promote senescence in PTEN null; p53 null human cancer cell lines and arrest tumorigenesis without enhancing senescence in normal PTEN wt cells. SKP2 inhibitors act by enhancing p27 levels, another essential player of senescence in tumors. These findings also demonstrate that PTEN deficient tumor cells are more susceptible that normal cells to SKP2 inhibitors and that PTEN deficient cells conserve the capability to undergo senescence even in presence of p53 mutations or deletions (Nardella et al. 2011).

8.3 Pro-senescence Therapy in Tumors: Pre-clinical and Clinical Development

8.3.1 Therapy-Induced Cellular Senescence

Therapy-induced cellular senescence (TIS) occurs in tumors in response to radiotherapy or selected chemotherapy agents and is a potential strategy for cancer treatment (Roninson 2003; Ewald et al. 2010). The mechanisms that mediate TIS in cancer cells are not well defined but are generally linked to DNA damage enhancement. Tumor cells can be forced into senescence by agents of clinical interest in the management of human cancers, such as docetaxel, bleomicina, cyclophosphamide doxorubicin, etoposide and cisplatin. Ionizing radiation can also induce senescence in different cancer cell lines (Chang et al. 1999; Roninson 2002; Gewirtz 2008). Interestingly, many evidences indicate that therapy-induced senescence may contribute to treatment outcome in vivo. Primary murine lymphomas have been shown to respond to cyclophosphamide therapy by engaging a senescence program controlled by p53 and p16INK4a (Schmitt et al. 2002). In addition to this, analysis of senescence markers in human biopsies from cancer patients after neoadjuvant chemotherapy revealed the occurrence of chemotherapy-induced senescence and its association to treatment outcome (te Poele et al. 2002; Roberson et al. 2005; Coppe et al. 2008). Therapeutically advantageous outcome might be achieved through the combination of TIS with treatments aimed to eliminate cancer senescent cells. On this line, recent findings indicate that senescence cells can be selectively targeted in murine cancers. Virotherapy has been lately explored to eliminate senescent cancer cells by introduction of oncolytic measles vaccine virus (MeV) (Weiland et al. 2014). In addition, Dorr and colleagues recently showed that senescence cells exhibit specific metabolic requirements that can be pharmacologically targeted to selectively eliminate these cells in vivo. This approach has been shown to improve treatment outcome in a model of TIS (Dorr et al. 2013).

8.3.2 p53 Targeting

Inactivation of p53 functions is an almost universal feature of human cancer cells (Lane et al., 2010b). However several cancers such as prostate cancer or glioblastoma conserve an intact p53 response even at advanced or metastatic stage (Lane et al., 2010b). The tumour-suppressive function of p53 predominantly relies on its function as a transcription factor and it can either positively or negatively regulate the expression of numerous target genes. Experimental models demonstrate that reactivation of p53 in established p53 null tumors leads to regression of lymphomas and sarcoma without affecting normal cells. The mechanism responsible for tumor regression depends on the tumour type, with the main consequence of p53 restoration being apoptosis in lymphomas and cellular senescence in sarcomas (Ventura et al. 2007). Another paper demonstrates that reactivation of p53 in established liver cancer results in tumor regression driven by senescence and activation of an anti tumor immune response (Xue et al. 2007). These and previous studies have inspired therapeutic approaches based on p53 reactivation. The most common approach to reactivate p53 in tumors is the p53 gene therapy. This approach is based on adenoviral delivery of p53 in tumor cell lacking p53. p53 gene therapy is now in widespread use in China (Shi and Zheng 2009), but it has not been approved in USA. Another strategy is activating the p53 through siRNA and antisense RNA's that block the function of the negative regulators Mdm2, MdmX, and HPV E6 (Jiang and Milner 2002; Zhang et al. 2005; Yu et al. 2006). However these approaches are not yet in clinical trial. The most promising approach to enhance p53 function in tumor cells, currently in the clinic, is the use of small molecules that activate p53 either directly or indirectly. There are several phase I clinical trials open with novel p53-mdm2 interaction inhibitors such as DS-3032b, SAR405838 and the Nutlins family members RO5503781, RO6839921, RO683992 (Table 8.1). These compounds show an increased tolerability for cancer patients compared to previous MDM2-P53 inhibitors that have showed excessive toxicity such as JNJ-26854165 (Wade et al. 2013). The compounds CP-31398 and APR-246 (PRIMA-1 analogue) (Peng et al. 2013) have been shown to enhance apoptosis by reactivating p53 function in cells with mutant p53. This approach has been developed to selectively treat tumors with mutant p53. APR-246 is now in clinical trial in combination with carboplatin in ovarian cancer (Table 8.1). Additional approach to reactivate p53 in cancer cells is targeting SirT1. This protein negatively regulates p53 by promoting deacetylation of p53 and its destabilization (reviewed in van Leeuwen and Lain (van Leeuwen and Lain 2009)). Inhibition of SirT1 can slow down tumor growth activating p53 function. There are several SirT1 inhibitor in pre-clnical trials such as e.g., sirtinol, suramin, tenovins, and 3, 2′, 3′, 4′-tetrahydroxychalcone) that can be administered in single, whereas others such as EX-527 and cambinol need concomitant addition of DNA damaging agents such as etoposide to have an effect on p53 (Lane et al., 2010b). Finally treatment with Src and c-Kit kinase inhibitor Dasatinib also resulted in enhanced p53 activity in human acute myeloid leukemia stem cells (Dos Santos et al. 2013). Further trials are needed to validate whether p53 and senescence are selectively enhanced in tumors treated with p53-targeting compounds.

Table 8.1 Pro-senescence cancer therapeutics in pre-clinical and clinical development

Function	Compound	Stage of development	Refs
Mutant p53 reactivation	CP-31398	Phase I	NCT00900614
Sir T1 inhibitors	Sirtinol	Preclinical	Ref. (Lane et al. 2010)
	Suramin, Tenovins 3,2′,3′,4′-tetrahydroxychalcone		
P53-MDM2 inhibitors	DS-3032b	Phase I	NCT01877382
	SAR450838		NCT01985191
	RO5503781		NCT01636479
	RO6839921		NCT01901172
	RO683992		NCT01773408
			NCT01462175
SCF-SKP2 complex inhibitor	MLN4924	Phase I	NCT00722488, NCT01011533, NCT00677170 and NCT00911066
CDK inhibitors	PD0332991	Phase I/II	NCT01821066
	LEE011	Phase I	NCT01953731
	LY2835219	Phase I	NCT01756781
	ZNF313	Preclinical	NCT01237236
			NCT01913314
MYC inhibitors	10058-F4 and its derivatives CPI-0610 (BET family proteins inhibitors)	Preclinical	Ref. (Huang et al. 2006)
			Ref. (Delmore et al. 2011)
Telomerase inhibitor	GRN163L (Imetelstat)	Phase I/II	NCT01256762
			NCT01137968
PTEN inhibitor	VO-HOPIC	Preclinical	Ref. Alimonti et al. 2010b)

8.3.3 Oncogene Inactivation

Cancer cells harbor many genetic alterations, but still remain dependent on the continued expression of a single aberrant oncogene (Sharma and Settleman 2007; Zuber et al. 2011). Interestingly, inactivation of MYC promotes tumor regression through cellular senescence in several diverse tumor types including lymphoma, osteosarcoma, and hepatocellular carcinoma (Wu et al. 2007). MYC influences the tumour microenvironment through different mechanisms, including activation of angiogenesis and suppression of the host immune response (Bellovin et al. 2013). Importantly, senescence resulting from MYC inactivation requires an intact immune system, as previously outlined (Nardella et al. 2011). Therefore, the development

and use of small molecules to inactivate MYC, to either target the protein for degradation or inhibit its activity (such as, 10058-F4 and its derivatives (Huang et al. 2006)) could also prove to be an efficient pro-senescence therapy. This strategy may be combined with an immuno-modulatory approach. RNAi technologies could also provide strategies to target MYC for cancer therapy and senescence induction (Pastorino et al. 2004; Vita and Henriksson 2006; Civenni et al. 2013). Therapeutic approaches aiming at the repair or suppression of these mutant oncogene products could be generally effective for the treatment of cancer. Interestingly CPI-0610, a novel BET protein bromodomain inhibitor in clinical development has been recently shown to suppress MYC transcription (Delmore et al. 2011) (Table 8.1). These compounds may be therefore used in MYC positive tumors to drive senescence by blocking c-MYC protein levels.

8.3.4 Therapeutic Modulation of Cell Cycle Machinery

The complex machinery of cell cycle in eukaryotes is controlled by a family of protein kinase complexes, wherein each complex is composed of a catalytic subunit, the cyclin-dependent kinase (cdk), and its essential regulatory subunit, the cyclin (Sherr et al. 1994; Jacks and Weinberg 1998). CDKs and cyclins drive the cell through the cycle. CDKs and cyclins drive the cell through the cycle. Expression of oncogenic Ras or loss of tumor suppressor genes induce cellular senescence, with high levels of the CDKs inhibitors p16INK4a, p15 and p21/CIP1 (Serrano et al. 1997; Lin et al. 1998). The discovery that CDKs inhibitors, particularly p21/CIP1, p16INK4a, and p27, accumulate in senescent cells and are essential for the senescence induction, led to the idea that compounds that enhance the levels of the CDKs inhibitors may be used for pro-senescence therapy for cancer. Although the induction of p16INK4A seems to be predominantly driven by transcription factors, the expression of p27 is strongly controlled through a balance of translation and proteasomal degradation. *SKP2* inhibitors can induce senescence through the accumulation of p27, in tumor lacking p53 pathway (Lin et al. 2010). A SKP1–CUL1–F-box protein (SCF)–SKP2 complex inhibitor, MLN4924, currently in Phase I clinical trials, now offers the possibility to investigate its potential to act as a pro-senescence therapy, through its ability to stabilize p27 in tumors (Table 8.1). In addition, p27 is rarely mutated in cancer, but reduced levels and mislocalization of p27 strongly correlate with poor prognosis. As a consequence, treatments that enhance p27 may be an opportunity for cancer therapy. A recent paper also demonstrates that CDK2 inactivation in MYC positive cells switch proliferation in senescence (Campaner et al. 2010), suggesting that CDK2 inhibitors may act as pro-senescence compounds in MYC positive tumors. Since there are several CDK2 inhibitors in clinical development (Senderowicz 2003), these compounds may represent a valid class of pro-senescence therapy for cancer in different patients. Treatments with CDK 4/6 inhibitors, such as PD0332991, also induce senescence (Leontieva and Blagosklonny 2013). These inhibitors can block Rb phosphorylation, thus arresting the cell cycle

(Capparelli et al. 2012; Leontieva and Blagosklonny 2013). Several CDK 4/6 inhibitors are currently under clinical evaluation. PD0332991 is in phase I-II of clinical trial and it has been tested alone or in combination with chemotherapy. LEE011 and LY2835219 are two additional CDK4/6 inhibitors in Phase I clinical trial (Table 8.1). Inhibition of a novel cell cycle activator ZNF313 (E3 ligase for $p21^{WAF1}$) profoundly delayes cell cycle progression and accelerates $p21^{WAF1}$-mediated senescence (Han et al. 2013). A microRNA, miR-519 has been found to repress tumor growth via multiple p21-inducing pathways by triggering autophagy (Abdelmohsen et al. 2012). Thus, targeting CDKs may alter one or more regulatory events resulting in restoration of cell-cycle checkpoints and may slow cell growth, induce apoptosis (Chen et al. 1999) or drive senescence and autophagy (Capparelli et al. 2012).

8.3.5 Telomerase Inhibition

Telomerase is a ribonucleoprotein that maintains the length of telomeres. It has been reported that the reactivation of the telomerase complex, which is normally silenced in somatic cells, is required for the transformation process and the progression of cancer. High levels of TERT and/or telomerase activity are common in cancer cells and are associated with poor prognosis in cancer patients (Gertler et al. 2004). There are several therapeutic approaches currently proposed that focus on targeting the telomerase complex. Among these different approaches, the specific inhibition of the enzymatic activity of telomerase may represent a powerful pro-senescence approach. GRN163L (Imetelstat) is a lipid-conjugated N3′→P5′ thio-phosphoramidate oligonucleotide that blocks the template region of telomerase (Nardella et al. 2011). This drug is currently in Phase II clinical trials and it holds promise as a strong anticancer agent. However it is unknown whether this compound can induce senescence in human tumor lesions (Table 8.1).

8.3.6 SASP Reprogramming

Cellular senescence is a dichotomic phenomenon. It is a barrier for cancer on one hand and on the other hand it can stimulate development of cancer via secretion of proinflammatory cytokines and proteases, thus acquiring a phenotype called senescence-associated secretory phenotype (SASP), that turns senescent fibroblasts into proinflammatory cells (Davalos et al. 2010). SASPs comprises of soluble signalling factors, chemokines, insulin-like growth factor-1, secreted proteases, tissue-type plasminogen activators, the uPA receptor, and the plasminogen activator inhibitors (Coppe et al. 2010). These factors have a potential impact on tissue microenvironments and they can stimulate tumor progression through induction of an EMT (Laberge et al. 2012a). Chemical screening protocol with a library of approved drugs was tested in normal human fibroblasts and lead to the identification

of glucocorticoids as potential suppressors of select components of the SASP (Laberge et al. 2012b). In another paper several known compounds and small molecule inhibitors have been shown to block senescence in pre-clinical trials, by interfering with the SASP (Acosta et al. 2013). These evidences highlighted the possibility that SASP reprogramming can be used to selectively target cancer cell and would have promising results. Interestingly the SASP has also the potential to activate and promote an anti-tumor response triggering tumor clearance (Kang et al. 2011). However, therapeutic intervention aiming at enhancing the SASP intensity or promote the upregulation of cytokines that mediate "paracrine" senescence have not been developed yet. A potential caveat of these treatments may originate from the fact that SASP has also been associated in certain contexts with pro-tumorigenic effects (Campisi and d'Adda di Fagagna 2007).

8.3.7 PICS Induction

Induction of senescence by targeting PTEN in cancer cells is provocative. This approach has been successfully used both in vitro and in vivo in a pre-clinical trial using the PTEN inhibitor VO-OHpic (Table 8.1). Targeting a potential tumor suppressor seems quite risky. However similar approaches have been successfully used in clinic to validate the concept of synthetic lethality. The use of PARP inhibitors, compounds that block the function of PARP, a gene involved in DNA repairs, have been proved to be effective in cells with BRCA1 mutations (Ashworth 2008). Given that the majority of tumors have PTEN monoallelic mutation or deletion, PTEN inhibitors may be used in clinic to induce senescence without significant risk for the patients. The temporary inactivation of PTEN activity in the tumor cells induces a senescence response through a signaling short circuit driven by hyper-activation of a PI3K–AKT–mTOR–p53 signaling pathway, as described above. By contrast, the effect of such inhibitors on wild-type cells that express PTEN at normal levels, results in only a transient decrease in PTEN activity leading to a marginal increase in the activation of the AKT–mTOR signaling pathway. Given that PTEN inactivation promote p16INKa activation by regulating the APC complex, inhibitors of PTEN may be also used in combination with CDK inhibitors such as SKP2 inhibitors or MDM2 inhibitors. However pre-clinical data supporting this hypothesis are still lacking (Nardella et al. 2011).

8.3.8 Metabolic Manipulation of Senescent Cells

Senescent cells, though not dividing, remain metabolically active and produce many secreted factors, that can either stimulate or inhibit the tumor growth (Shay and Roninson 2004). Altered cell metabolism is a key feature of the senescence phenotype and also contributes to successful execution of the senescence program

(Kuilman et al. 2010). Interestingly, a strong increase in energy metabolism has also been observed in therapy-induced senescent tumors. Dorr and collegues recently showed that lymphomas that were rendered senescent by treatment with chemotherapy, exhibited an increased glucose transporter and glycolytic enzymes expression, and consequent higher glucose uptake (Dorr et al. 2013). Of note, senescent cells were selectively susceptible to inhibition of glucose transporters, and were therefore targetable for selective elimination. In addition to glucose metabolism, also glycogen metabolism has been linked to senescence. It has in fact been shown that depletion of the catabolic enzyme glycogen phosphorylase results in glycogen accumulation, that is associated with reduced proliferation and a corresponding induction of senescence (Favaro et al. 2012). To further confirm the connection existing between cell metabolism and senescence, Kaplon and colleagues recently showed that OIS evoked through BRAF or KRAS overexpression was accompanied by higher rate of oxygen consumption, augmented pyruvate oxidation and increased mitochondrial oxidative metabolism. These results were also confirmed on Braf-overexpressing melanoma cells in vivo. Interestingly, OIS was dependent on PDH activation both in vitro and in vivo, thus rendering this enzyme a key regulator of senescence induction and a potential barrier against malignant transformation. Finally a recent study revealed that the connection between metabolism and senescence is mediated by p53. Downregulation of malic enzymes modulates p53 activation, thereby leading to a strong induction of senescence, but not apoptosis, whereas enforced expression of malic enzyme suppresses senescence (Jiang et al. 2013). All together these evidences highlight the potential to target the senescence-related metabolic conditions to selectively eliminate senescent cells in vivo, thus leading to tumor regression and improved treatment outcomes.

8.4 Summary and Future Directions

Senescence has been proved to be an essential tumor suppressive barrier to counteract tumor progression in vivo. Several pre-clinical evidences demonstrate that senescence enhancement in tumors, also called “pro-senescence therapy” for cancer, is effective in promoting tumor regression. In vitro and in vivo studies using several chemotherapeutic drugs demonstrate that these compounds can induce senescence and apoptosis. However senescence induction by chemotherapeutic drugs may also result in side affects by enhancing DNA damage in normal cells. Hence, further studies are required to identify drugs that are able to induce senescence in tumor cells rather than in normal cells. Pro-senescence target therapies are a promising class of compounds, some of which are currently tested in clinical trials. A better understanding of the signaling mechanisms involved in senescence and senescence by-pass would improve the selection of compounds with pro-senescence activity to be used in the clinic. Induction of PICS offers several therapeutic benefits over OIS, as absence of hyperproliferation and DDR, thus preventing genomic instability and the risk of secondary mutations. In addition to this, PICS induction

may help to induce senescence in quiescent cancer-initiating cells (CICs), which contribute to the maintenance of the tumor in many cancer types. In the light of this evidence, full-proof screening platforms aimed to discover novel pro-senescence compounds able to provoke PICS, would aid the development of more efficient treatment modalities.

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Chapter 9
Integrating Proteotoxic Stress Response Pathways for Induction of Cell Death in Cancer Cells: Molecular Mechanisms and Therapeutic Opportunities

Kristopher S. Raghavan, Robert Clarke, and Ayesha N. Shajahan-Haq

Abstract The endoplasmic reticulum (ER) is a major organelle that is involved in protein synthesis, and in proper maintenance of cellular homeostasis and adaptation to adverse environments. Perturbations in the cellular environment is sensed by transmembrane ER resident proteins to initiate an intricate and highly conserved signal transduction pathway called the unfolded protein response (UPR). The central objective of the UPR is to prevent the accumulation of unfolded/misfolded proteins in the UPR and adapt to cellular stress by promoting cell survival. However, if a threshold is exceeded by the stress, the UPR can trigger programmed cell death pathways. The ability of the UPR to maintain survival has important implication in human diseases such as cancer. Cancer cells can up-regulate signaling associated with the UPR to promote growth and resist anti-cancer therapy. Knowledge of the mechanism associated with the UPR may provide novel therapeutic targets for cancer therapy.

Keywords Apoptosis • Extrinsic apoptosis pathway • Intrinsic apoptosis pathway • Cellular stress • Endoplasmic reticulum • Unfolded protein response • Drug resistance • Cell death • Autophagy • Necrosis • IRE1α • GRP78 • PERK • CHOP • ATF6

9.1 Introduction

The ER is the key intracellular organelle responsible for protein and lipid biosynthesis, protein folding and trafficking, calcium homeostasis, and several other vital processes. The ER, is therefore, equipped with coping pathways to allow the cell to adapt to environmental stress such as the UPR pathway (Schroder and Kaufman 2005). Perturbation of ER functions can result in aggregation of improperly

K.S. Raghavan • R. Clarke • A.N. Shajahan-Haq (✉)
Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, 3970 Reservoir Rd., NW, NRB W405B, Washington, DC 20057, USA
e-mail: ans33@georgetown.edu

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folded (unfolded/misfolded) proteins post-translation, which can result from various cellular stimuli including hypoxia, nutrient deprivation or cytotoxic agents (Clarke et al. 2011; DuRose et al. 2006; Feldman et al. 2005). Accumulation of unfolded proteins is sensed by three known transmembrane proteins of the ER. While only the inositol-requiring enzyme-1 (IRE1) pathway has been found in yeast (Back et al. 2005), metazoans use two additional pathways, double stranded RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Okada et al. 2002). The UPR triggers transcriptional and translational responses culminating in up-regulation of glucose regulated protein 78, also known as heat shock protein 70, or binding immunoglobulin protein(GRP78, HSPA5,BiP) to promote protein folding (Li et al. 2008), global inhibition in protein synthesis to reduce protein load, and potentiation of ER-associated degradation (ERAD) to eliminate unfolded proteins from the ER (Buck et al. 2007).

The tumor micro-environment of solid tumors, as well as low in pH and nutrients. Cancer cells can promote survival and adapt to the ER stress induced by these conditions by transducing transcriptional and translational mechanisms mediated by the UPR. On the other hand, following prolonged stress, if recovery is not achieved, cell death can be also be initiated by the UPR. In this chapter, major components of the UPR and strategies to target these molecules in anti-cancer therapy in cancer progression will be discussed.

9.2 The Unfolded Protein Response

Following mRNA translation, completion of protein synthesis requires the translated polypeptide chain to be folded into its proper conformation within the ER. Once the protein is folded, it is transported through the Golgi apparatus to the location within the cell in which it will be useful when active. The folding process requires various chaperone proteins and folding enzymes to achieve stability of the polypeptide chain in its sterochemically favored form. However, this process is not resistant to error since part of the polypeptide chain may remain unfolded or certain segments of amino acids may become incorrectly folded (Malhotra and Kaufman 2007a, b). These unfolded proteins are not trafficked out of the cell and begin to accumulate in the ER, causing the ER to become distended; a distinct sign of ER stress. If this stress remains unresolved, it will result in a positive feedback loop of more improperly folded proteins, which will further impair other cellular processes. The continued formation and accumulation of these non-functional proteins in the ER creates stress that can result in decreased cellular metabolism and protein synthesis due to the waste of valuable cellular resources such as amino acids and metabolites. Furthermore, ATP levels can be depleted, and DNA and Protein damage can occur due to an increase in the reactive oxygen species (Malhotra and Kaufman 2007a, b). The potential for severe cellular damage caused by the accumulation of unfolded proteins is too great a threat, and thus, the process of UPR has evolved to alleviate cellular stress and restore cellular homeostasis (Rutkowski et al. 2006; Wu and Kaufman 2006). The function of UPR is twofold: (1) attenuate

production of new proteins to prevent accumulation of defective proteins, and (2) facilitate the elimination and recycling of the accumulated unfolded proteins in the ER. The former is accomplished through multiple modes of action including the targeted degradation of mRNA transcripts to prevent translation, slowing the transcription of new mRNA, and by preventing newly synthesized proteins (whether properly or improperly folded) from entering the lumen of the ER (Malhotra and Kaufman 2007a, b). Once the incoming stream of unfolded proteins has been tapered, the UPR sets out to alleviate the ER of its improperly folded protein aggregates. Because the severity of the defects of the unfolded proteins vary, the UPR firsts attempts to rectify the folding errors so that it will not need to expend unnecessary energy degrading a protein when it could more easily fix it instead. To this effect, the UPR increases cellular concentrations of foldases, chaperones, and various other effectors to aid in re-folding or repairing the accumulated proteins (Schroder and Kaufman 2006).

When the UPR has exhausted all options to salvage a protein, it is targeted for elimination. This process involves a degradation pathway known as endoplasmic reticulum-associated degradation (ERAD) (Meusser et al. 2005). There are two types: in ERAD (I), which involves an ubiquitin-mediated proteasome pathway, and ERAD-II which is an autophagic/lysosomal pathway (Fujita et al. 2007). The type of ERAD that the cell employs is dependent on the protein to be degraded. ERAD (I) is used for soluble proteins that are transported out of the ER into the cytoplasm where they are ubiquinated and then reduced to basic metabolites by the proteasome (Buck et al. 2007). Conversely, ERAD (II) initiates a cannibalistic cellular process called autophagy (discussed below) to degrade those unfolded proteins that are insoluble (Rusten et al. 2008).

9.3 Stress Sensors at the Plasma Membrane of the ER

The ER possesses various transmembrane receptors which will detect the presence of unfolded proteins as they begin to accumulate within the ER (Schroder and Kaufman 2006; Shamu and Walter 1996) (Fig. 9.1). There are three main sensors: protein kinase RNA-like endoplasmic reticulum kinase (PERK, gene: EIF2AK3), inositol-requiring protein-1α (IRE1α, gene: ERN1), and activating transcription factor 6 (ATF6) (DuRose et al. 2006). In the absence of stress, under basal conditions, these sensors are maintained in an inactive state due to their luminal domains being bound to GRP78 (Li et al. 2008; Todd et al. 2008). Once the stress sensors detect unfolded proteins beginning to collect in the ER, GRP78 dissociates from the sensors and then binds to hydrophobic domains on the unfolded proteins (Knarr et al. 1995) in order to attempt a repair of the protein (Hur et al. 2006). The cellular concentration of free GRP78 regulates the activity of the three branches of UPR, and changes in this concentration can alter the regulation of UPR (Hendershot et al. 1994). In the case of the IRE1a branch of the UPR, GRP78 is an adjustor for the sensitivity to certain stresses and will alter the sensitivity of IRE1α activity when returning to homeostasis in the ER. Due to its crucial role in ER homeostasis,

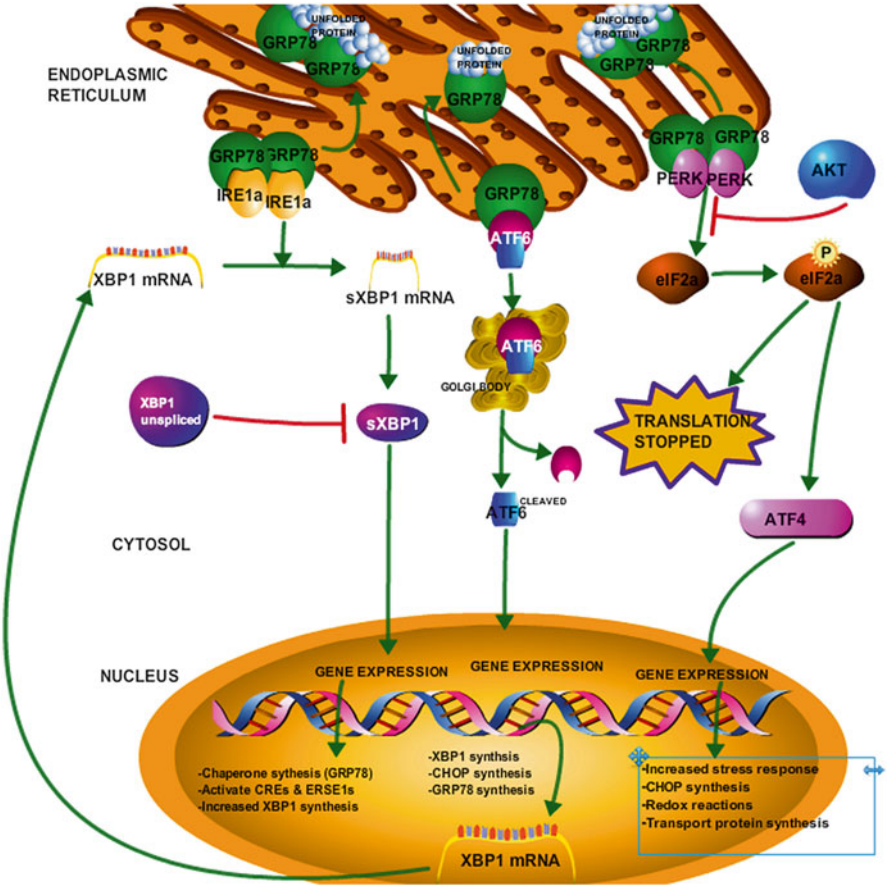

Fig. 9.1 The three main signaling pathways of UPR. UPR begins in the ER. GRP78 binds the main stress response proteins, IRE1α, ATF6, and PERK in an inhibitory fashion under normal conditions. When ER senses stress, GRP78 dissociates from the proteins under its charge to bind the unfolded proteins and chaperone them to be degraded, thus releasing and activating IRE1α, ATF6 and PERK. PERK dimerizes, autophosphorylating and facilitates the phosphorylation of eIF2a (this process can be inhibited by AKT as illustrated by the *red arrow*). A phosphorylated eIF2a proceeds to stop further protein translation, as well as activate the transcription factor ATF4. ATF4 enter the nucleus, binds DNA and promotes the gene expression of various proteins to increase the stress response, including CHOP, transport proteins, and proteins to decrease the amount of reactive oxidative species (ROS). When ATF6 dissociates from GRP78 it is still inactive and translocates to the Golgi apparatus where it is cleaved to its active form and then translocated into the nucleus and functions as a transcription factor, to promote gene expression of CHOP, additional GRP78, and XBP1 mRNA. The XBP1 mRNA exits the nucleus and is spliced by the active IRE1a into a shorter form, sXBP1 mRNA which is then expressed into the protein sXBP1. sXBP1(which can be inhibited through the activity of normal length XBP1 as illustrated) enters the nucleus as a transcription factor to promote the increased effectiveness of the stress response in a positive feedback loop including the synthesis of additional XBP1, GRP78, and other stress response proteins

expression levels of GRP78 protein are often used as a biomarker of ER stress. It is also important to note that GRP78 overexpression can be a negative regulator of UPR (Okamura et al. 2000). Below, we focus on each branch of UPR to explain specific mechanisms and also their interdependence.

9.3.1 IRE1α

IRE1α is a dual function protein with both kinase and endoribonuclease properties (Yoshida et al. 2001). While the IRE1α variant is ubiquitously expressed, the IRE1β variant is predominantly expressed in intestinal epithelial cells (Schroder and Kaufman 2006). As mentioned previously, the primary goal of the UPR is to resolve ER stress and maintain homeostasis. However, sustained stress can prompt UPR target genes to promote programmed cell death. During ER stress, IRE1α is dephosphorylated and its endoribonuclease activity is attenuated to prepare the cell for a pro-death outcome (Lin et al. 2007). IRE1α is capable of activating the apoptotic-signaling kinase 1 (ASK1) and Jun-N-terminal kinase (JNK) that promote apoptosis (Urano et al. 2000). On the other hand, the trans-autophosphorylated (active) role of IRE1α can be viewed in a pro-survival context. Active IRE1α promotes the splicing of a critical pro-survival gene called X-box binding protein 1(XBP1) (Yoshida et al. 2001). Splicing of XBP1 is considered "unconventional" due to the fact that the majority mRNA splicing is regulated by splicosomes within the nucleus, whereas XBP1 splicing is regulated through the endoribonuclease activity of IRE1α in the cytoplasm (Yoshida et al. 2001). During this splicing process, a 26 nucleotide intron is removed. This creates a frame-shift which encodes a larger version of XBP1 (sXBP1) which functions as a transcription factor (Feldman et al. 2005). sXBP1 translocates to the nucleus to activate transcription of cytoprotective genes two ways: (i) by its ability to activate specific cAMP response elements (CREs) (Clauss et al. 1996), and (ii) through ER stress response elements (ERSE1) (Oyadomari and Mori 2004). sXBP1 also upregulates the expression of GRP78, as well as additional XBP1 (Calfon et al. 2002). Research has also supported that the over-abundance of unspliced XBP1 actively works to suppress the activity of its spliced counterpart, which suggests that the relative balance between XBP1 and sXBP1 could be a significant factor in determining the ultimate fate of a cell (Lee et al. 2003).

XBP-1 may promote human breast carcinogenesis through impairment of cell differentiation regulation (Fujimoto et al. 2003). High levels of sXBP1 is associated with poor outcome (Davies et al. 2008) and antiestrogen resistance in estrogen receptor positive breast cancer (Gomez et al. 2007). Recently, sXBP1 expression has been associated with progression of triple-negative subtype, an aggressive form of breast cancer (Chen et al. 2014). Therefore, XBP1 is a potential target in anti-cancer drug development (Shajahan et al. 2009).

9.3.2 PERK

PERK contains a cytosolic protein kinase domain and is activated through trans-autophosphorylation and homodimerization. PERK activation inhibits further protein translation, thus, slowing down the influx of both folded and unfolded proteins into the ER adding to the stress (Harding et al. 2000). Activated PERK attenuates translation by phosphorylating eukaryotic translation initiation factor-2α (eIF2α) to prevent the assembly of the 80s-ribosome that is required to perform protein translation, therefore, halting the process of protein synthesis (Shi et al. 1998). Not all new protein translation is prevented since important cytoprotective UPR target genes and their transcription factors still need to be synthesized. To enable synthesis of these pro-survival proteins, phosphorylated eIF2α also selectively promotes the translation of specific mRNAs that possess small open reading frames on their 5′ untranslated region (Harding et al. 1999). Additionally, PERK allows the selective translation of specific proteins that can potentially resolve the ER stress, such as increasing p53 levels in the cell (Zhang et al. 2014), which is controlled by a ribosomal-Hdm2 that prevents the hdm2-mediated ubiquitination and degradation of p53. Increased levels of p53 allow greater control over the cell cycle, which in turn allows the cell to adequately repair and/or replicate its DNA properly. Furthermore, PERK mediated attenuation of translation can activate the avian reticuloendotheliosis virus-T leukemia (Rel) family transcription factors, including the pro-survial molecule, NF-kB (Schroder and Kaufman 2006).

Activating Transcription Factor 4 (ATF4) is another protein that is translated following PERK activation (Harding et al. 2000). Synthesized ATF4 enters the nucleus to induce gene expression of transcriptional regulators involved with various functions that will either help the cell relieve the ER stress and return the cell to homeostasis, or induce apoptosis. Such gene products produced include those involved in correcting the redox status of the cell, cellular metabolite synthesis and transport, and the pro-apoptotic protein C/EBP homologous protein, CHOP (also known as growth arrest- and DNA damage- inducible gene 153, GADD153) (Vattem and Wek 2004). CHOP is an important player in the induction of apoptosis when ER stress is unable to be resolved and will be discussed later in the chapter. Interestingly, PERK can be inhibited through AKT phosphorylation, which would prevent the phosphorylation of eIF2α, and thus shutting this branch of the UPR signaling cascade. Further investigation into this pathway has shown that inhibition of PERK in tumor cells can induce cell death (Mounir et al. 2011). Collectively, PERK and its downstream effector, eIF2α, fulfill a pro-survival role in UPR.

9.3.3 ATF6

Upon detection of ER stress, ATF6 is released from GRP78 and is translocated to the Golgi apparatus. There, it is cleaved by two proteases, separating its cytoplasmic domain with its membrane bound domain. There, the serine protease site-1 protease (S1P)

cleaves ATF6 in its luminal domain. Consequently, metalloprotease site-2 protease (S2P) then cleaves ATF6 in the transmembrane domain and releases its bZIP transcription factor domain into the cytosol (Schroder and Kaufman 2006). The cleaved version of ATF6 is then translocated into the nucleus where it binds the ER stress response element $CCAAT(N)_9CCACG$ to initiate the gene expression of UPR target genes (Haze et al. 1999). Such target genes include GRP78, CHOP, and XBP1, in addition to other genes that code for proteins needed to help with protein folding, transport, and degradation (Okada et al. 2002).

9.4 Cell Death Mechanisms

Prolonged ER stress can lead to cellular suicide in order to protect the integrity of the system it belongs to. Thus, activation of UPR is closely connected to activation of programmed cell death pathways. Programed cell death is a complex and vital cellular function. These pro-death pathways are composed of several varied mechanisms which will be detailed below. The distinct hallmark of cancer includes the cellular ability to resist or subvert cell death (Hanahan and Weinberg 2000). Indeed, cancer cells acquire various mechanisms to escape or block the induction of programmed cell death. Large amounts of research have been carried out to investigate these cell death mechanisms to better understand these pathways in carcinogenesis. There are three main modes of programmed cell death: apoptosis, autophagy, and necrosis.

9.4.1 Apoptosis

Apoptosis (also known as Type I Programmed Cell Death) is a vital aspect of numerous processes, including cell turnover, immune system activity, embryonic development, and most relevant to cancer, chemical/radiation-induced cell death (Fig. 9.2). Apoptosis, an energy-dependent process, is characterized by loss of mitochondrial membrane potential, plasma membrane "blebbing, (bulging irregularly)" cell shrinkage, and nuclear fragmentation (Hotchkiss et al. 2009). There are many facets to the mechanisms of apoptosis, and expectantly, these mechanisms involve signaling cascades complete with sophisticated feedback responses. In cancer, apoptosis is particularly important and it is induced by therapeutic agents to reduce or eliminate malignant growths.

9.4.2 The Intrinsic Pathway of Apoptosis

There are currently two main pathways in apoptosis: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is initiated when specific pro-apoptotic signals such as irreparable DNA damage or other serious stresses cause mitochondrial outer membrane permeabilization (MOMP), opening protein channels to allow

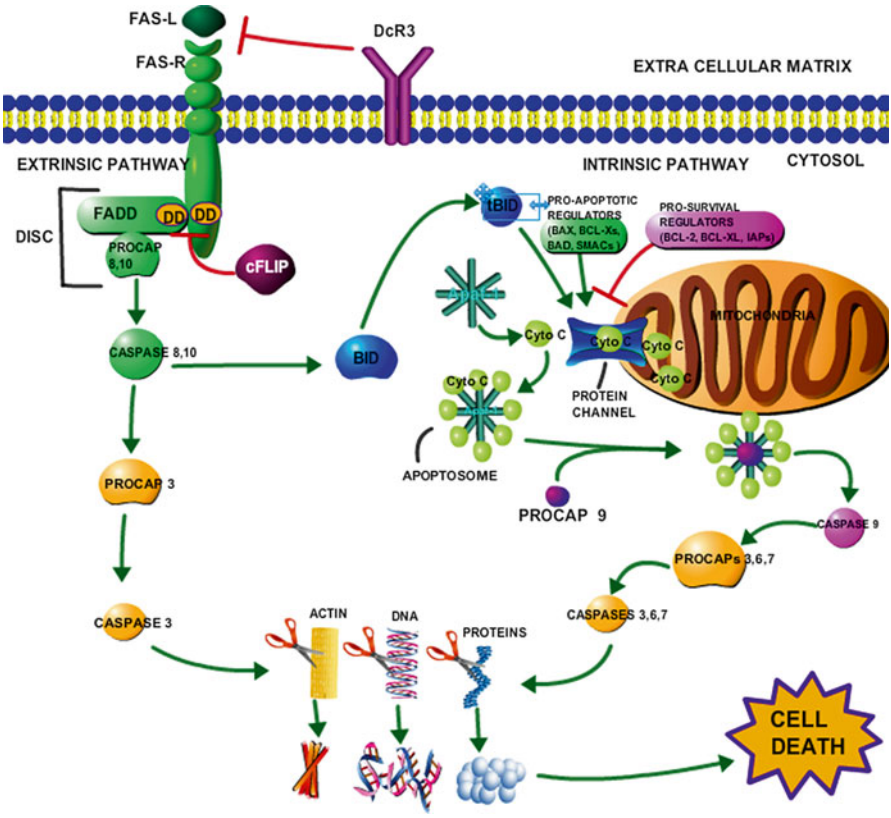

Fig. 9.2 Intrinsic and extrinsic apoptotic pathways and their convergence. The following shows the cascade of events that occur in each form of apoptosis. In the extrinsic pathway we use the FAS(R) receptor for an example, however, the same series of events occurs through the stimulation of other death receptors (TRAIL, TNF DR4/5) and their respective ligands and adaptors. Upon ligand binding, FADD docks with the receptor at the shared death domain (DD),thereby recruiting procaspase 8, 10 forming (DISC). Procaspase 8 and 10 are cleaved from FADD and activated into caspase 8, 10 (the initiator caspases). Caspase 8, 10 perform two functions: they cleave BID into a tBID which goes on to be used in the intrinsic pathways, and they cleave procaspase 3 into its active form caspase 3 (the executioner caspase). Caspase 3, which is also the executioner caspase in the intrinsic pathway proceeds to degrade many cellular materials including, actin in the cytoskeleton, DNA and other nuclear proteins, cytoplasmic proteins and organelles until the cell is so unstable that it causes a systematic failure and death. The extrinsic pathway has a few negative regulators illustrated with the red inhibition arrows; DcR3 competitively binds the FAS ligand to prevent the whole signaling cascade, as well cFLIP prevents the docking of FADD to the DD of the FAS receptor, preventing the activation of procaspase 8, 10. Within the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP), triggers the assembly of pro-apoptotic regulators, and the deactivation of pro-survival(apoptotic inhibiting) regulator to create protein channels that allow the release of cytochrome c (Ctyo C) from the intermembrane space. Apaf-1 binds cyto c to form the apoptosome (forming a wheel-like shape), which binds and activates procaspase 9 into caspase 9(initiator caspase). Procaspase 9 activates the executioner caspase 3, 6, 7 to fulfill the same role that they fulfill in the extrinsic pathway, thus causing cell death

the release of cytochrome c, a highly soluble heme protein and electron carrier found in between the mitochondria's inner and outer membranes employed in the electron transport chain (Tafani et al. 2002). Cytochrome c forms a wheel-like complex with apoptotic protease activating factor-1(Apaf-1) creating the "apoptosome." Procaspase 9, the initiator caspase (family of potent cysteine-dependent, aspartate-specific proteases), is bound by the apoptosome and modified to its enzymatically active form, caspase 9. Caspase 9 will then cleave the executioner procaspases, procaspase-3, 6, and 7, thus converting them to their active caspase form. These active executioner caspases cleave cellular substrates and catalyze the fragmentation and degradation of DNA, the cytoskeleton, and nuclear proteins, as well as the formation of apoptotic bodies, cross-linking of proteins, ligand expression for phagocytic cell receptors, and finally the uptake of degraded cellular material by phagocytic cells (Elmore 2007). The intrinsic apoptotic pathway is tightly regulated by a large family of proteins known as the B cell lymphoma 2 (Bcl-2) family which consists of at least 25 genes that contain up to 4 specific Bcl-2 Homology (BH1-4) domains that mediate hetero-and homodimerization with each other. This family consists of proteins with wide variability in function, an can be arranged based on how they regulate apoptosis; whether they perform a pro-apoptotic function (i.e. Bcl-X_s, Bax, Bak, Bim) or an anti-apoptotic function(i.e. Bcl-2, Bcl-X_L, Bcl-w, Bfl-1). During homeostasis, these regulators, in tandem with other regulatory mitochondrial proteins known as small mitochondria-derived activators of caspases (SMACs) and their counterparts, inhibitors of apoptosis proteins (IAPs) (Fesik and Shi 2001), often hold each other in check. As a result, the fate of the cell is largely determined by the balance in the activation of these pro-death and pro-survival proteins (Karam et al. 2007). In many cancers that attempt to escape Bcl-2 mediated apoptosis, the pro-survival regulators will be overexpressed (Bcl-X_L) and/or pro-death regulators (Bax) will be inhibited. The expression patterns of certain regulators can even help to predict the efficacy of certain treatments; for example, expression of Bcl-2 can predict the outcome of radiotherapy in laryngeal cancer with an accuracy of 71 % (Nix et al. 2005).

9.4.3 The Extrinsic Pathway of Apoptosis

The extrinsic pathway is initiated at the plasma membrane via the ligand binding and activation of cell surface death receptors (Ashkenazi and Dixit 1998). There are several death receptors (Fas, Trail-R1, TNFR), all with their own domain adaptors (FADD, TRADD) and ligands (FasL, Trail, TNFα); one main death receptor is Fas (also called FasR or apoptosis antigen 1) whose corresponding ligand and adaptor domain is FasL and Fas Associated Death Domain (FADD), respectively (Wajant 2002). These adaptors are recruited via their specific death domains to the receptor upon ligand binding and are necessary for facilitating the assembly of the Death Inducing Signaling Complex (DISC) which comprises procaspases 8 and 10, waiting to be cleaved and activated to then initiate the same execution pathway observed

in the intrinsic pathway. In this pathway, FADD and DISC are homologous to Apaf-1 and the apoptosome from the intrinsic pathway respectively. Several decoy death receptors (DcRs), members of the tumor necrosis factor receptor (TNFR) superfamily can compete with the legitimate death receptors for ligand binding to avoid apoptosis (Zong et al. 2014). These decoy receptors are often overexpressed in cancer (Huang et al. 2014). Another inhibitory mechanism exists through the ability of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) to bind the death domain of FADD (and other adaptors), preventing the binding of the initiator pro-caspases 8 and 10, and thus inhibiting apoptosis (Salvesen and Walsh 2014).

The intrinsic and extrinsic pathways of apoptosis are linked. While both of these pathways are initiated uniquely, they both converge on the same execution pathway. Additionally, member of the Bcl-2 family of apoptotic regulators, BH3 Interacting Domain (Bid) function in both pathways. In the extrinsic pathway, when capsase 8 becomes activated, it cleaves cytoplasmic BID into a truncated form (tBid) which translocates to the mitochondria to help facilitate the release of cytochrome c to stimulate increased apoptosis via the intrinsic pathway (Brasacchio et al. 2014).

9.4.4 Autophagy

Autophagy (Type II Programmed Cell Death) is a cannibalistic process of self-degradation of cytoplasmic materials, translating quite literally to "self-eating" (Fig. 9.3). Autophagy is identified by the swelling of the vacuole to massive proportions within the cell, loss of organelles, and the appearance of many autophagosomes (Kroemer and Levine 2008). There are three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (the type that is most commonly referred to plainly as "autophagy") (Jin et al. 2013). This process has long been thought to be a broad-scope, non-selective process, however, recently several forms of selective autophagy have been identified.

CMA is one such selective form of autophagy which applies most appropriately to UPR in that improperly folded proteins in the cytoplasm are systematically targeted and directly translocated into the lysosome to be degraded. This translocation is mediated by Heat Shock Cognate 70 (HSC70 aka HSPA8) as well as lysosome-associated membrane protein 2(LAMP2) (Dice 2007).

Microautophagy can be both selective and non-selective. Microautophagy describes the process by which the lysosome directly consumes and degrades cytoplasmic materials. Selective forms of this type include microautophagy of the mitochondria (micromitophagy), peroxisomes (micropexophagy), and the nucleus (Krick et al. 2008). While microautophagy and macroautophagy employ separate signaling pathways, some of the autophagy-related proteins are required in both processes (Li et al. 2012).

Macroautophagy (referred to hereafter simply as, autophagy) requires the formation of multi-membrane organelles known as autophagasomes to engulf cytoplasmic

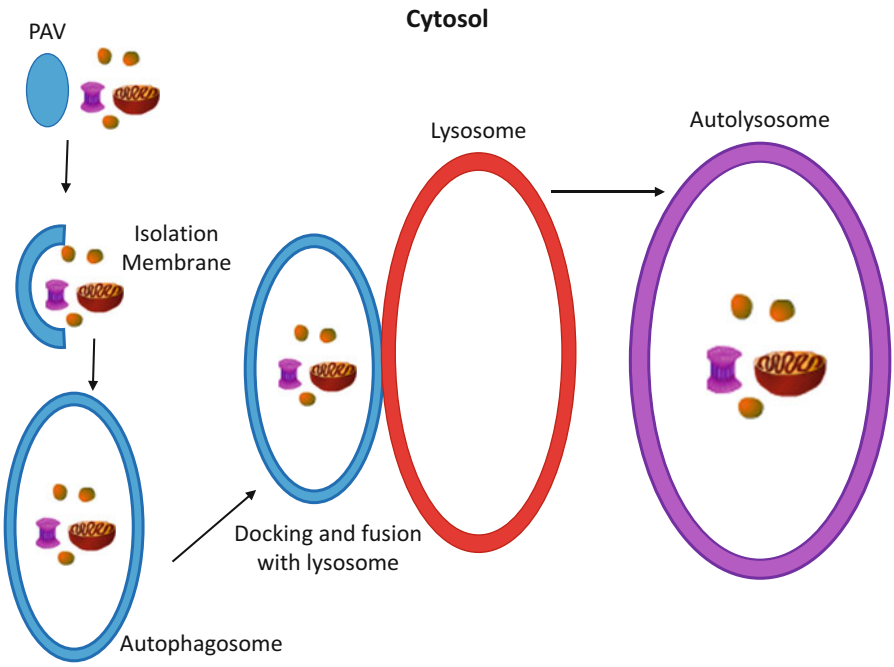

Fig. 9.3 The progression of autophagy. Autophagy begins with the pre-autophagic vesicle (PAV) and the cellular materials (i.e. misfolded proteins, ribosomes, mitochondria, etc.) that have been selected to be broken down into their raw materials. The PAV begins to elongate to create the isolation membrane, which will begin to engulf the targeted materials to eventually encase them in the autophagosome. The autophagosome will then traffic the cellular material to the lysosome (or vacuole; same process) where it will dock with the membrane of the lysosome. As this process continues, the autophagsome expels its contents into the lysosome and fuses with the membrane further, creating the autolysosome. The cellular materials are digested and reduced to their raw metabolites for use elsewhere in the cell

materials. These autophagasomes will transport the cellular material destined to be degraded to the lysosome whereby the autophagasome will fuse with and disperse its contents into the lysosome. While non-selective forms of autophagy exist, there are many forms of selective autophagy including: ribophagy (ribosomes), mitophagy (mitochondria), pexophagy(peroxisomes), glycophagy (glycogen), lipophagy (lipid droplets), xenophagy(foreign pathogens), zymophagy (secretory molecules), and perhaps most relevant to this chapter on UPR, ER-phagy. ER-phagy is induced to prevent the excessive distension of the ER during stress caused by the aggregation of unfolded proteins (Yorimitsu and Klionsky 2007). Indeed, researchers have observed the formation of autophagasomes simultaneously with the induction of UPR (Bernales et al. 2006). Interestingly, there has been research that shows that the degradation of cellular materials during autophagy occurs in a certain order; starting with cytosolic and proteosomal proteins proceeding further to organelles (Kristensen et al. 2008).

In general, the events that require the formation of autophagosomes, engulfing of cellular material, and docking to the lysosomes are controlled through the unimaginatively termed, autophagy-related proteins (ATGs), encoded via the ATG genes (Maiuri et al. 2007). The initiation of autophagy is controlled by ATG1, ATG13, and ATG17; the induction of this process requires a signal from the nutrient sensing protein, mTOR, which catalyzes the dephosphorylation of ATG13 which forms a complex with ATG1 and ATG17. The formation of the autophagosome proceeds through to vesicle nucleation and the formation of an isolation membrane controlled via ATG6 (aka Beclin-1). Once this occurs, mTOR and BCL-2 in the ER must be suppressed before ATG6 and ATG13 can form the autophagosome. The vesicle then elongates to consume target cytoplasmic materials; this is achieved through the action of several ATG proteins including: ATG3 ATG4, ATG5, ATG7, ATG10, ATG12, and ATG16. After the autophagosome has been assembled, the autophagosome will dock with the lysosome, fusing membranes and allowing the contents of the autophagosome to enter the lysosome to be degraded by lysosomal enzymes (Maiuri et al. 2007).

Autophagy is an important albeit unclear subject in the scope of cancer research. Researchers propose that autophagy fulfils a pro-survival role due to the necessity to overcome stresses such as nutrient depletion and hypoxia (Hanahan and Weinberg 2000). However, autophagy is a cell death mechanisms responsible for the extermination of cancer cells. Autophagy can have a profound effect on the efficacy of chemotherapeutic treatments and the development of resistance to anti-cancer therapies. When autophagy is inhibited, the therapeutic responses of resistant cancer cells to chemo-, endocrine- or radio-, therapies is increased, further supporting the pro-survival role of autophagy in cancer cells (Cook et al. 2011, 2012; Schwartz-Roberts et al. 2013). Autophagy is also instrumental in the cross-talk between the tumor-microenviroment and the cancer cells themselves, specifically in the syncing of metabolic activity (Pavlides et al. 2012).

Pro-survival autophagy is closely connected to the UPR. When the integrity of protein folding is compromised, and the continuous misfolding of proteins leads to energy depletion in the cell, UPR initiates and signals an up-regulation of autophagy in an attempt to recycle proteins to generate new metabolites and ATP, ideally alleviating the stress (Clarke et al. 2011, 2012; Cook et al. 2011, 2012). It is important to note that the nature of the autophagic response is not so dramatic as to be switched-on or switched-off, rather it is gradual; the level of autophagy increases or decreases to appropriate itself with the current level of stress (Tyson et al. 2011). However, what complicates autophagy in cancer is that although it is generally accepted as a pro-survival mechanism, excessive or prolonged stress will inevitably trigger cell death by arresting autophagy, which can in turn induce apoptosis or necrosis (Crawford et al. 2010; Schwartz-Roberts et al. 2013). An intricate relationship is emerging between autophagy and apoptosis, but unfortunately, there is little consensus among researchers. For instance, it is unclear whether what we describe as autophagic cell death is a truly novel form of cell death executed solely through autophagy, or if instead we are witnessing a tandem effort of autophagy and apoptosis in which the former simply initiates the latter. There are several hypotheses based

on different cell lines and models. In the case of true autophagic cell death, the total volume of the autophagic vesicles would be equal or greater than the amount of free cytosol, thus a huge proportion of cytoplasmic material as well as organelles would be destroyed; this would cause irreversible damage to the cell, inducing atrophy and failure of cellular functions still intact leading inevitably to the death of the cell (Lum et al. 2005). In the scenario where autophagy triggers other forms of cell death, autophagy is first initiated as a response to stress stimuli, however, when the stress is not able to be resolved, apoptosis or necrosis is triggered (Espert et al. 2006).

9.4.5 Necrosis

Necrosis (Type III Programmed Cell Death) is a much less selective process, and is commonly associated with injury induced by physical trauma. Necrosis causes inflammation in groups of cells and is found commonly in the center of solid tumors. It is characterized by organelle and plasma membrane swelling, and rupture (Hotchkiss et al. 2009). It was long thought that necrosis was not regulated through signaling pathways and was only a passive form of cell death, however, research in the last decade has proved otherwise. Necroptosis, a programmed form of necrosis dependent on receptor-interacting protein kinase-3 (RIPK3), has been identified and is seen to be mediated in the cell through the binding of Tumor Necrosis Factor (TNF) to its cellular receptor (TNFR) which binds to and activates specific cell death receptors(Galluzzi and Kroemer 2008). Precise mechanism of the regulatory pathway of necroptosis remain largely undiscovered, however, recent research suggest that it is related to the other forms programmed cell death since necroptosis can be induced when both autophagy and apoptosis are blocked (White 2008).

9.5 UPR in Drug Resistant Cancer

As we have detailed above, UPR in a normal cell is a vital function for maintaining homeostasis and keeping the cell healthy. Unfortunately, cancer cells can take over this protective mechanism for their own survival, and to enable themselves to adapt to cellular stress and escape programmed cell death. As stated previously, hypoxia is a source of ER stress which is commonly found in solid tumors; UPR is commonly seen to be up-regulated in solid tumors, helping to relive the hypoxic stress and contributing to a poor clinical prognosis due to the tumor's ability to withstand radio- and chemotherapy (Koumenis and Wouters 2006). UPR also helps cancer cells survive stresses such as genomic instability caused by a dysregulated cell cycle, nutrient and energy depletion, but most importantly in the clinical setting, DNA damaging anti-tumor therapeutics. Indeed, cancer that is successful in overhauling its UPR mechanism will become significantly more resistant to therapy (Clarke et al. 2012; Cook et al. 2012). Additionally, UPR can induce a quiescent-like state

in which the cell growth is arrested, yet survival remains competent, to allow them to resist drug/radiation induced stress (Ranganathan et al. 2006).

There has been a great deal of research hinged on understanding this form of acquired resistance in tumors, and more importantly, finding ways to circumvent the resistance to restore sensitivity to therapeutics. In research on hepatocellular carcinoma (HCC), a particular cancer biomarker, CD147, was identified as an inducer of UPR closely associated with the expression of GRP78 (which is also seen to be overexpressed in many malignant diseases (Cook et al. 2012). When CD147 was up-regulated, apoptosis in HCC was inhibited, and a decreased sensitivity to Adriamycin (chemotherapeutic agent) was observed; thus CD147 (or other UPR-related proteins) inhibition presents itself as an encouraging opportunity to improve the efficacy of anti-cancer therapeutics (Tang et al. 2012). In human epidermoid carcinoma (HEp3) cells, cell cycle regulator p38 plays a role in the activation of PERK and up-regulation of GRP78; moreover the latter is required to facilitate the inhibition of Bax (a pro-death regulator of apoptosis) activation to promote cell survival and drug resistance (Ranganathan et al. 2006). Further research into the role of GRP78 in cancer treatment showed that the forced overexpression of GRP78 conferred resistance to topoisomerase inhibitors (apoptosis inducers) across various tumor cell types (Reddy et al. 2003).

9.6 Conclusions and Future Perspectives

The UPR is a highly conserved adaptive mechanism that is instrumental in maintaining cellular homeostasis in response to ER stress in normal cells. In cancer, up-regulation of the UPR can protect the cell from ER stress resulting from therapeutic interventions. While the precise mechanisms of the UPR activation in cancer development remains unknown, it is clear that targeting pro-survival players in the UPR pathway can help promote cell death in various cancer cell and tumor models. Investigating novel therapeutic targets in the UPR pathway is an active area of research in cancer (Table 9.1). As we understand the complexity of the UPR and the programmed cell death pathways, it is likely that combination therapies that induce pro-death but inhibit the pro-survival pathways of the UPR and autophagy will be most effective to inhibit survival in cancer cells. Moreover, the activation of specific arms of the UPR is likely to be dependent on the cellular context, and therefore, knowledge of the signaling mechanism associated with the specific cancer type is needed. In summary, the UPR is a promising yet perplexing area of cancer research. More research is needed to understand the role of the resilience of UPR in averting cell death and promoting cancer growth in human body.

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Table 9.1 Drugs (both pre-clinical and clinical) targeting UPR and cell death pathways

Drug	Pathway	Target	Action	Reference
Sunitinib	UPR	Multiple kinases	Inhibits IRE1 activity	Bouchecareilh et al. (2011)
Sorafenib	UPR	Multiple kinases	Activation of UPR	Magnaghi et al. (2013)
HKH40A	UPR	GRP78	Downregulates GRP78 expression	Kosakowska-Cholody et al. (2014)
Toyocamycin	UPR	IRE1	Inhibits XBP1 mRNA splicing	Kawamura et al. (2008)
GSK2656157	UPR	PERK	Inhibits PERK phosphorylation	Atkins et al. (2013)
Bortezomib	UPR	26S Proteosome (active in apoptosis)	Activates PERK	Mimura et al. (2012)
MAL3-101	UPR	HSP70	Induction of XBP1 mRNA splicing	Goloudina et al. (2012)
17-AAG	UPR/Autophagy	HSP90	Inhibit HSP90	Jhaveri et al. (2012)
Doxorubicin	Apoptosis	Topoisomerase II	BCL2 family regulation	Minotti et al. (2004)
Cisplatin	Apoptosis	DNA	Caspase activation, p53 activation, cytochrome c release	Jamieson and Lippard (1999)
Paclitaxel	Apoptosis	Microtubule stabilization	Caspase activation, phosphorylation of BCL2 and BCL-X, JNK activation, CD95/FAS expression, autophagy	Schiff and Horwitz (1981)
Chloroquine	Autophagy	Lysosome	Block lysosomal acidification/degradation of autophagosome and contents	Egger et al. (2013)
Sirolimus/rapamycin	Autophagy	mTOR	Inhibit mTORC1; induce autophagy	Chan (2004)

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Chapter 10
The Hsp70 Family of Heat Shock Proteins in Tumorigenesis: From Molecular Mechanisms to Therapeutic Opportunities

Anna Budina-Kolomets, Subhasree Basu, Lili Belcastro, and Maureen E. Murphy

Abstract The HSP70 family of molecular chaperones consists of at least eight members that are highly evolutionarily conserved. Whereas more than one member of this family is implicated in cancer, the most compelling and abundant data point to the involvement of the predominant stress-inducible form of this protein in cancer etiology and progression. High levels of HSP70 staining in tumors emerged as a significant marker of poor prognosis in human tumors over 20 years ago. Since that time, the important role of this protein in cellular transformation, viral infection, immune function, and the cellular stress response has come to be appreciated and understood. In the past 10 years, the findings that many different types of human tumors are addicted to this protein for survival, and that silencing HSP70 is cytotoxic to tumor but not normal cells, have led to the emergence of the first specific inhibitors of this family of molecular chaperones for cancer therapy. Here-in we review the pro-tumorigenic function(s) of this protein, our understanding of how HSP70 mediates protein quality control, and the current efforts to target and inhibit this protein for cancer therapy.

Keywords HSP70 • HSP90 • Apoptosis • Apoptosome • Chaperone • Co-chaperone • Drug resistance • HSP70 inhibitors • Allosteric inhibitors of HSP70 • Proteostasis • Senescence • Lysosome • Autophagy • Cancer therapy

10.1 HSP70 Expression in Cancer

The stress-inducible isoform of HSP70 (also called HSP701A1, HSP70-1, or HSP72) is a molecular chaperone that is overexpressed in the majority of human cancers, of various histological origins. Conversely, this protein is nearly

A. Budina-Kolomets • S. Basu • L. Belcastro • M.E. Murphy, Ph.D. (✉)
Program in Molecular and Cellular Oncogenesis, The Wistar Institute,
3601 Spruce St, Philadelphia, PA 190104, USA
e-mail: mmurphy@wistar.org

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undetectable in normal, unstressed cells (Daugaard et al. 2007; Murphy 2013). Elevated basal expression of HSP70 in cancer cells is believed to help these cells to maintain protein homeostasis in the high stress environment of cancer. This stress is derived from both extrinsic (for example, the exterior tumor microenvironment) and intrinsic factors within the cell. Specifically, the tumor microenvironment subjects cancer cells to excess reactive oxygen species, acidosis, hypoxia, and nutrient deprivation; these signals in turn activate the stress-inducible transcription factor heat shock factor 1 (HSF1), the primary transcription factor regulating HSP70 expression (Zorzi and Bonvini 2011). Additionally, intrinsic factors such as aneuploidy, the presence of mutant oncoproteins and high metabolic stress can lead to increased HSF1 activation and HSP70 expression in tumor cells. Finally, there are also some regulators of HSF1, such as the key metabolic controller mTOR, which are frequently activated in cancers, and lead to overexpression of HSP70 in tumor cells (Chou et al. 2012). HSP70 expression promotes growth and survival of tumors in the presence of such adverse conditions (Leu et al. 2011; Juhasz et al. 2013; Lee et al. 2013). Overexpression of HSP70 in cancer has been related to tumor growth, differentiation, drug resistance, metastasis, apoptosis, and poor patient prognosis (Stankiewicz et al. 2005; Juhasz et al. 2013; Kang et al. 2013; Lee et al. 2013).

10.2 High Levels of HSP70 Are Associated with Poor Prognosis

The majority of evidence suggests that HSP70 overexpression correlates with adverse prognosis, decreased differentiation, and increased proliferation (Ciocca and Calderwood 2005). HSP70 expression has been correlated with both early and late stages of cancer. For example, high levels of HSP70 correlates with early stages of disease in prostate (Abe et al. 2004) and hepatocellular (Chuma et al. 2003) carcinomas. Correspondingly, studies involving the ectopic expression of HSP70 in untransformed cells have shown that HSP70 confers tumorigenicity in vitro and in vivo, suggesting that induction of high HSP70 levels is an early event in tumor formation (Seo et al. 1996; Barnes et al. 2001; Meng et al. 2011). There are also multiple studies correlating HSP70 expression with advanced stages of disease. For example, overexpression of HSP70 serves as a marker of advanced disease in melanoma (Lazaris et al. 1995), oral squamous cell carcinoma (Kaur et al. 1998), bladder cancer (Syrigos et al. 2003), intestinal gastric cancer (Lee et al. 2013), colorectal cancer (Hwang et al. 2003), and uterine squamous cell carcinoma (Ralhan and Kaur 1995). Importantly, overall and disease-free survival is significantly reduced when HSP70 is overexpressed in several cancers including endometrial carcinoma (Nanbu et al. 1998), acute myeloid leukemia (Thomas et al. 2005), and breast cancer (Ciocca et al. 1993; Thanner et al. 2003). Furthermore, high levels of HSP70 have been associated with drug resistance. Chemotherapeutic drugs cause increase expression of HSP70 as the cells launch a

cytoprotective response. As two examples, an in vitro study of prostate cancer found that treatment with cisplatin increased the expression of HSP70 and mediated resistance to apoptosis (Ren et al. 2008). Similarly, imatinib-resistant chronic myeloid leukemia cells demonstrated a threefold increase in HSP70 expression compared to imatinib-naive cells, and HSP70 knockdown significantly reduced cell viability in drug-resistant cells (Pocaly et al. 2007).

10.3 Cancer-Relevant Pathways Controlled by HSP70

10.3.1 Apoptosis

The accumulated evidence indicates that cancer cells become addicted to HSP70 due to the ability of this protein to serve as a master regulator of several cancer-relevant pathways. For example, HSP70 is able to inhibit extrinsic and intrinsic apoptosis at several points in the apoptotic cascade by binding and modulating stress kinases, and by interfering with the function of the BCL-2 family of apoptotic proteins (Arya et al. 2007; Zorzi and Bonvini 2011). This protein also inhibits the extrinsic pathway of cell death by inhibiting receptor-mediated activation of apoptosis. It does this by binding directly to the death receptors DR4 and DR5 that are activated by TNF-related apoptosis-inducing ligand (TRAIL), which inhibits the formation of the death-inducing signaling complex (DISC) (Guo et al. 2005) (Fig. 10.1a). In general, activation of c-Jun N-terminal kinase (JNK) under stress normally results in the simultaneous phosphorylation and inhibition of the anti-apoptotic proteins BCL-2 and BCL-X_L, and phosphorylation and activation of the pro-apoptotic proteins BID and BAX. However, HSP70 has been shown to inhibit stress-induced cell death by binding and inhibiting apoptosis signal-regulated kinase 1 (ASK1), JNK, and p38 mitogen activated protein kinase (Zorzi and Bonvini 2011; Juhasz et al. 2013). Therefore, in the presence of HSP70, BCL-2 and BCL-X_L remain active and stabilize the mitochondria, BID and BAX remain inactive and do not translocate to the mitochondria, and SMAC and cytochrome c are not released (Stankiewicz et al. 2005) (Fig. 10.1a). Downstream of the mitochondria, HSP70 binds to the caspase recruitment domain of apoptosis protease-activating factor-1 (APAF-1), and thereby prevents the recruitment of procaspase-9 and the formation of the apoptosome (Ravagnan et al. 2001) (Fig. 10.1a). Correspondingly, HSP70 depletion with small interfering RNA (siRNA) in pancreatic cancer cells led to increased Annexin V-positive cells and caspase-3 and caspase-9 activation, which is consistent with observations that HSP70 can prevent caspase-9 recruitment to the apoptosome (Aghdassi et al. 2007). Given these multiple avenues of controlling cancer cell apoptosis, it is perhaps not surprising that infection of cancer cells with an adenovirus expressing antisense HSP70 results in loss of viability in vitro and tumor reduction in vivo (Nylandsted et al. 2002).

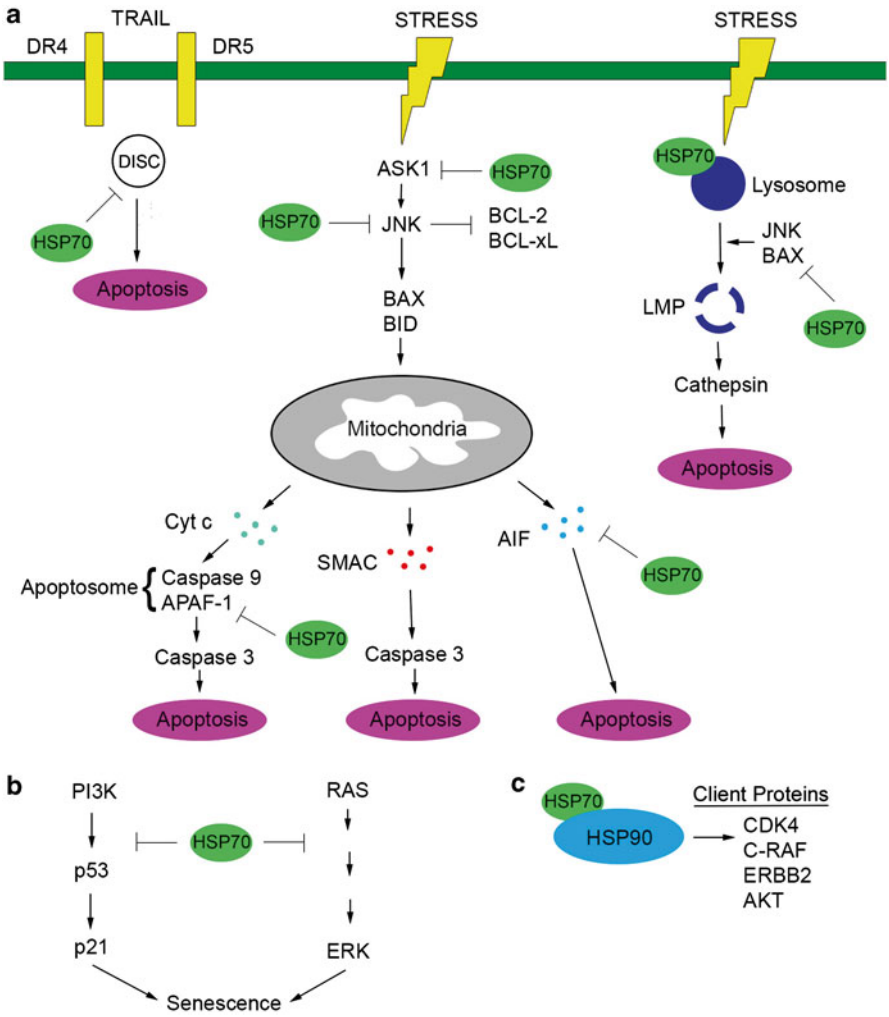

Fig. 10.1 Overexpression of HSP70 affects multiple cancer-relevant pathways. (**a**) HSP70 inhibits the extrinsic apoptosis pathway by binding to the death receptors DR4 and DR5, and preventing the formation of the *DISC*. *HSP70* inhibits the intrinsic pathway by inhibiting ASK1 and JNK, thereby preventing BAX and BID translocation to the mitochondria. HSP70 also prevents apoptosis downstream of the mitochondria by binding to APAF-1 and preventing formation of the apoptosome. In addition, HSP70 stabilizes lysosome membranes and inhibits lysosome membrane permeabilization (LMP). (**b**) HSP70 inhibits both p53-dependent and -independent senescence. (**c**) As an obligate co-chaperone for HSP90, HSP70 is essential for the proper functioning of HSP90, and proper folding of HSP90 client proteins

10.3.2 Senescence, Lysosome Function

In addition to suppressing key proteins in the apoptotic pathway, evidence suggests that HSP70 can also inhibit senescence. For example, siRNA-mediated depletion of HSP70 results in a senescent phenotype, including flattened cell morphology, increased phosphorylation on serine 15 of p53, decreased proliferation, and increased senescence-associated β-galactosidase staining (Yaglom et al. 2007). Additionally, expression of oncogenic forms of PI3K and RAS in breast epithelial cells showed dependency on HSP70 for transformation, in both a p53-dependent and -independent manner, respectively (Fig. 10.1b) (Gabai et al. 2009). Lysosome stability is important for tumor growth, and lysosomal membrane permeabilization (LMP) induced by various stresses results in cell death, while extracellular release of lysosomal proteases can promote tumor invasion (Bivik et al. 2007; Doulias et al. 2007; Dudeja et al. 2009; Zorzi and Bonvini 2011). Specifically in cancer but not normal cells, HSP70 has been found bound to the lysosomal membrane, stabilizing it in the face of stressors such as tumor necrosis factor (TNF), etoposide, UV radiation, and hydrogen peroxide; notably, when HSP70 is depleted, these stresses result in increased cell death (Nylandsted et al. 2004; Bivik et al. 2007; Doulias et al. 2007). In addition, HSP70 interacts with proteins involved in lysosomal membrane permeabilization (LMP), including BAX, JNK, and p53, and this protein can prevent LMP and the release of proteolytic hydrolases, and to thus maintain cellular integrity.

10.3.3 HSP90

The chaperones HSP70 and HSC70 are obligate co-chaperones for HSP90. Not surprisingly then, depletion or inhibition of HSP70 results in reduced solubility of HSP90 client proteins, which become sequestered and inactivated in detergent insoluble compartments in the cell (Leu et al. 2011; Murphy 2013). In a well-controlled study by Workman and colleagues, simultaneous silencing of HSP70 and heat shock cognate 70 (HSC70) resulted in proteasome-dependent degradation of the HSP90 cancer-critical client proteins, C-RAF and CDK4, and initiation of apoptosis in cancer cells, but not in normal non-transformed cells (Powers et al. 2008). These data argue that simultaneous targeting of both HSP70, and the constitutively expressed family member HSC70, may be required for effective cancer therapy.

10.4 HSP70 Chaperone Structure

As indicated above, HSP70 is implicated in the pathogenesis of cancer (Ciocca and Calderwood 2005; Powers et al. 2009) and neurodegenerative diseases (Patury et al. 2009; Evans et al. 2010). Therefore, targeting HSP70 function represents a novel

therapeutic strategy for these diseases. This has led to increased interest in understanding the structure and mechanism of allostery of this chaperone. The Hsp70 family of proteins (HSP70 in humans, DnaK in *E. coli*) has a highly conserved domain structure. Each contains a 45 kDa N-terminal nucleotide binding domain (NBD) that has ATPase activity and a 25 kDa C-terminal protein substrate binding domain (SBD), joined by a short flexible linker (Bukau and Horwich 1998; Mayer 2010; Zuiderweg et al. 2013) (Fig. 10.2a). The interdomain linker is only 12 residues in length and is highly conserved (Vogel et al. 2006). Because these two domains are rarely seen together in crystal structures, their detailed structures were described separately in the 1990s. The NBD consists of two flexible lobes, I and II, and each is further divided into subdomains A and B, which form a deep nucleotide-binding cleft (Bork et al. 1992). Structural data suggest that the nucleotide-binding site is located at the bottom of the cleft (Flaherty et al. 1990). This structure was first reported for the NBD isolated from bovine ADP-bound HSC70, and subsequently has been confirmed by others (Flaherty et al. 1990; Harrison et al. 1997; Sriram et al. 1997; Chang et al. 2008).

The substrate binding domain (SBD) consists of two functionally relevant subdomains: SBDβ, a 15 kDa β-sandwich subdomain comprised of two four-stranded β sheets with a hydrophobic pocket for peptide binding between them, and SBDα, a 10 kDa mobile α-helical C-terminal region that is believed to function as a "lid" that closes over the substrate (Wang et al. 1998) (Fig. 10.2a). This structure was first described in *E. coli* for the SBD of DnaK in complex with a short peptide (Zhu et al. 1996). C-terminal to the "lid" is the least conserved region amongst HSP70 family members, which terminates with an EEVD (amino acid designations) motif, capable of interacting with tetratricopeptide repeat (TPR) domain-containing proteins. Sequence variation of this region is believed to allow different HSP70 family members to interact with distinct co-chaperones.

10.5 The HSP70 Functional Cycle

HSP70 assists the folding of its clients using repeated cycles of binding and releasing of misfolded proteins. The ability of the SBD to preferentially bind to exposed hydrophobic polypeptide sequences allows this domain to recognize the non-native states of many proteins (Chiti and Dobson 2006; Eichner and Radford 2011). In some cases, members of the DnaJ/HSP40 family of co-chaperones recognize and bind to such substrates first, and then present them to the HSP70 protein (Bukau and Horwich 1998; Han and Christen 2003). Similar to other ATP-dependent chaperones, the activity of HSP70 requires the energy of ATP binding and hydrolysis and involves critical allostery between the NBD and SBD. Specifically, ADP-ATP exchange in the NBD, which is catalyzed by nucleotide exchange factors (NEFs) like BAG-1 (Liu and Hendrickson 2007; Schuermann et al. 2008), triggers the attachment of the interdomain linker and the α-helical "lid" to the NBD, thereby opening up the peptide binding pocket (this is referred to as an "open" domain-docked

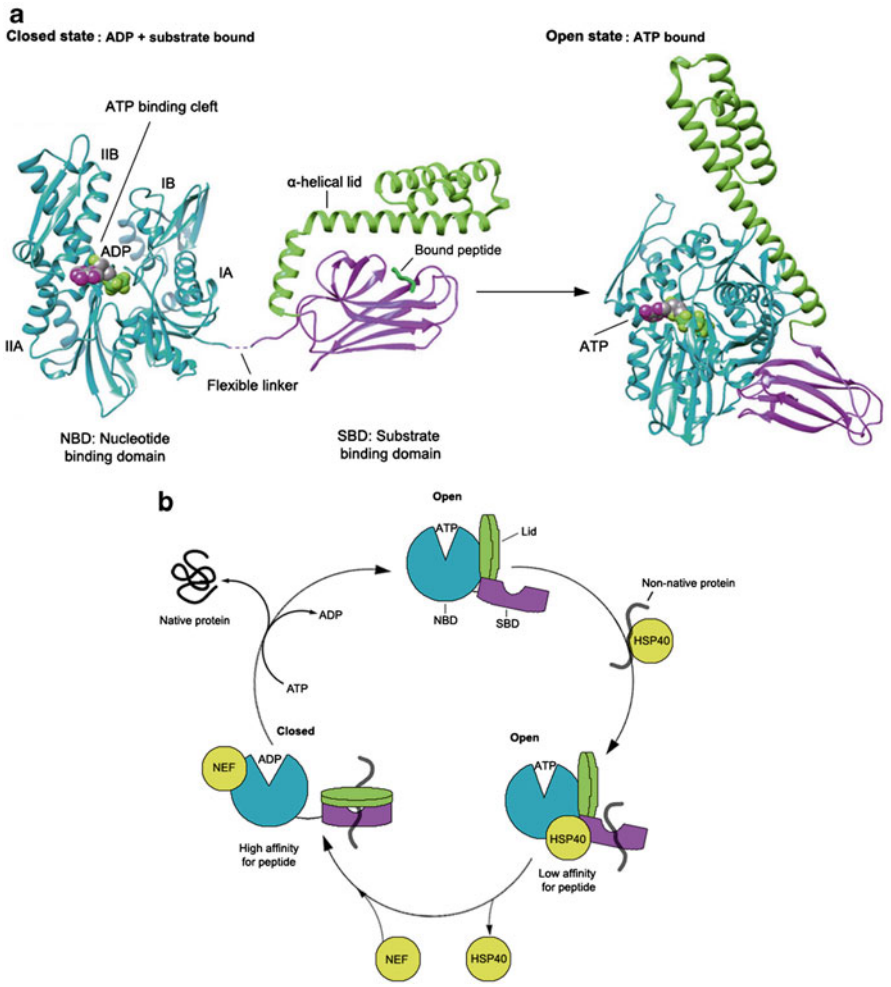

Fig. 10.2 Structure and allosteric cycle of HSP70. (**a**) The proposed confirmations of the two 'end-point' states of HSP70: the closed state is illustrated by the crystal structure of ADP-bound DnaK (*Escherichia coli* HSP70; PDB 2KHO; *right panel*); the open state is illustrated by the crystal structure of the ATP-bound state of Sse1 (yeast Hsp110; PDB 2QXL; *left panel*). (**b**) ATP binding to the nucleotide binding domain (NBD) facilitates low affinity binding of substrates, such as those presented to HSP70 via the HSP40 co-chaperone. HSP40 stimulates nucleotide hydrolysis and increases the affinity for substrate, by mediating the closure of the SBD α–helical lid. Nucleotide Exchange Factors (NEFs) assist with ADP release, which causes substrate release

conformation of HSP70) (Fig. 10.2b). Subsequently, this results in release of bound substrate from the SBD. Released unfolded peptides are believed to then spontaneously collapse into their native state in free solution (Sharma et al. 2010), or to re-bind to HSP70 for further unfolding, or to transfer to downstream chaperones and possibly be targeted for degradation. Binding of a new peptide to the SBD

induces a conformational change that is propagated back to the NBD, increasing the rate of ATP hydrolysis. Finally, hydrolysis of ATP is signaled back to the SBD, resulting in closing of the α-helical "lid" and enhancement of substrate affinity (that is, the "closed" domain-undocked conformation of HSP70) (Fig. 10.2b).

In the attempt to understand the allosteric regulation of HSP70, researchers have focused on comparing the crystal structures of ADP- and ATP-bound DnaK, an HSP70 orthologue from *E. coli* that shares about 60 % sequence homology with human HSP70 and has a similar domain structure. These studies revealed that in ADP-bound DnaK, the NBD and SBD behave as independent units, and the structures observed show no stable communication between them (Swain et al. 2007; Bertelsen et al. 2009) (Fig. 10.2a, *left panel*). However, upon ATP binding, the protein undergoes large conformational changes in both domains, which come together to form an intimately packed domain-docked structure (Fig. 10.2a, *right panel*) (Kityk et al. 2012; Zhuravleva et al. 2012; Qi et al. 2013). It was shown that the interdomain linker of DnaK binds between the IA and IIA subdomains of the isolated NBD, and this alone is necessary and sufficient for ATPase activation (Vogel et al. 2006; Swain et al. 2007). Similarly, the recently described structure of an HSP70 homolog, yeast HSP110, Sse1, shows that the linker is hidden between the NBD and SBD when HSP70 is in the ATP-bound state. This suggests a tight NBD-SBD interaction and is consistent with previously observed ATP-mediated conformational changes (Buchberger et al. 1995; Rist et al. 2006; Liu and Hendrickson 2007; Mapa et al. 2010). These structural data highlight the role of the interdomain linker in the docking of the SBD onto the NBD; however they do not explain how binding of ATP induces allosteric "lid" opening and subsequent loss of affinity for substrates (Flynn et al. 1989; Schmid et al. 1994). To understand the mechanism of this allosteric signal transmission between the NBD and SBD, Mayer and colleagues captured and characterized a normally transient conformation of human HSP70 bound to ATP by engineering disulfide bonds between SBDα and the subdomain IB of the NBD. They showed that ATP binding to the N-terminus of HSP70 induces a clockwise rotation of NBD lobe II, leading to closure of the nucleotide-binding cleft between subdomains IB and IIB. This conformational change widens a space between subdomains IA and IIA, creating a binding site for the interdomain linker, which then docks SBDβ to the ATP-stabilized NBD. The intimate interaction between the NBD and SBDβ interfaces results in SBDα displacement from SBDβ and binding to subdomain IB of the NBD ("lid" opening). Conformational changes of the SBD also propagate to the other side of SBDβ (loop L3,4 of the β-domain), where the substrate-binding cleft opens up, allowing bound substrates to rapidly dissociate. These dramatic allosteric rearrangements in both domains triggered by ATP binding lead to conversion of HSP70 into the stable, open domain-docked state (Kityk et al. 2012; Zhuravleva et al. 2012; Qi et al. 2013). This study reveals that open (ATP-bound) conformation of HSP70 differs from the closed (ADP-bound) not only in the orientation of the α-helical lid, but also in the conformation of the peptide-binding pocket of SBDβ. It also identifies the NBD-linker interaction as a first critical step in interdomain communication. Combined with the knowledge that mutations in the linker abolish chaperone

function of HSP70 (Han and Christen 2001), these data point to a role for the linker as a key site in allosteric regulation.

10.6 Opportunities to Interfere with the HSP70 Function

Increased understanding of how ligands modulate the HSP70 allosteric cycle facilitates the design of small allosteric inhibitors of HSP70 for therapeutic targeting of this chaperone. ATP hydrolysis plays a significant role in regulating the HSP70 chaperone activity. Therefore, inhibition of the enzymatic activity of HSP70 with competitive nucleotide analogs that disrupt HSP70-ATP interaction represent a logical approach to interfere with the function of this chaperone. While this strategy has been successful for HSP90 (Messaoudi et al. 2008), compounds that bind directly to the ATPase domain of HSP70 were not identified until recently. A lack of progress in targeting this domain can be explained by its unique structural and chemical characteristics. HSP70 has a 300-fold higher affinity for nucleotide binding when compared to HSP90 (Borges and Ramos 2006; Williamson et al. 2009; Massey 2010). In addition, analysis of the ATP-site, combining structure-based design and computational modeling, revealed that the ATP-binding site of HSP70 consists predominantly of hydrophilic residues, and that the interaction energy between the nucleotide and the chaperone is mostly mediated by the phosphate groups (Hurley 1996; Liu and Hendrickson 2007). This is generally associated with poor druggability of the enzyme active site because it does not allow the formation of hydrogen bonds and hydrophobic interactions between most inhibitors and the ATP-binding pocket (Halgren 2009). Despite these barriers, the ATP-binding site of HSP70 has a favorable size and is well enclosed, indicating that it remains a potentially druggable site. Indeed, recently novel competitive inhibitors of the HSP70 ATPase activity were identified, and these inhibitors were shown to inhibit cancer cell viability in vitro (Williamson et al. 2009).

Another way to modify HSP70 activity is by inhibiting its interactions with important co-chaperones, such as J proteins, nucleotide exchange factors (NEFs) and TPR domain-containing proteins. This can be achieved by blocking protein-protein interactions (PPIs) between HSP70 and co-chaperones or by targeting allosteric sites that disrupt these interactions. Normally, binding of a particular co-chaperone to HSP70 results in activation of a specific function in chaperone biology such as protein trafficking and translocation, protein folding or degradation (Table 10.1). Consequently, the rationale for developing chemical modulators of these interactions is to target a specific subset of HSP70 biology without impacting global proteostasis. Although PPIs are very difficult to inhibit (Arkin and Wells 2004; Gestwicki and Marinec 2007), several molecules have already been reported to specifically disrupt the interaction of HSP70 with particular co-chaperones and lead to distinct effects on cellular homeostasis (Yi and Regan 2008; Roodveldt et al. 2009; Dorard et al. 2011). Taken together, the available structural data suggest there are many opportunities to chemically target HSP70. Recent promising advances in developing HSP70 inhibitors are discussed below.

Table 10.1 Essential components of the HSP70 chaperone machinery

Co-chaperones	Function
J domain proteins	1. These interact with HSP70 at lobes IA and IIA of the Nucleotide Binding Domain (NBD) and the adjacent linker region to stimulate ATP hydrolysis and subsequently increase affinity for substrate binding by HSP70
	2. J proteins can specifically recognize and bind misfolded substrates and present them to HSP70 for refolding
	These interact with HSP70 at lobes IB and IIB of the NBD to catalyze the exchange of ADP to ATP, which results in lid opening and substrate release
Nucleotide Exchange Factors (NEFs)	HSP110 has a similar domain structure to HSP70. It functions as a "holdase" for non-native proteins
Hsp110	Interacts with lobe II of the NBD of HSP70 and releases nucleotide upon binding
HSPBP1 (Hsp70 binding protein 1)	Contains a C-terminal "BAG" domain that binds to lobes IB and IIB of the NBD of HSP70, which causes ADP release
BAG (BCL-2-associated AthanoGene) domain proteins	Has a ubiquitin-like domain that regulates the fate of HSP70-bound substrates
BAG-1, Bcl-2-associated athanogene 1;	Substrate-specific regulation of HSP70 clients for inhibition or increase of proteasomal degradation
BAG-2/3, Bcl-2-associated athanogene 2/3	Induced by HSF1, may play a role in tumor formation, stabilizes oncogenes and suppresses apoptosis in complex with HSP70
Tetratricopeptide repeat (TPR) domain-containing proteins	Bind to the C-terminus of HSP70 at the EEVD motif
CHIP	A ubiquitin E3 ligase, directs ubiquitination of HSP70-bound substrates for proteasome degradation
HOP	Mediates the association of HSP70 and HSP90, thereby allowing transfer of substrates

10.7 HSP70 Inhibitors

Recently, several inhibitors of HSP70 have been reported. Some of the most promising ones are discussed below, and their structures are depicted in Fig. 10.3a. For a more detailed comparative analysis of all the HSP70 inhibitors, readers are directed to some excellent recent reviews on this subject (Patury et al. 2009; Evans et al. 2010; Powers et al. 2010; Goloudina et al. 2012; Murphy 2013).

10.7.1 Deoxyspergualin/Dihydropyrimidines

One of the first compounds identified to bind and inhibit HSP70 function was the antibiotic 15-deoxyspergualin (DSG) (Plowman et al. 1987). DSG binds to the C-terminus of HSP70 (Fig. 10.3b) with a K_D of 4 μM and hinders its function by

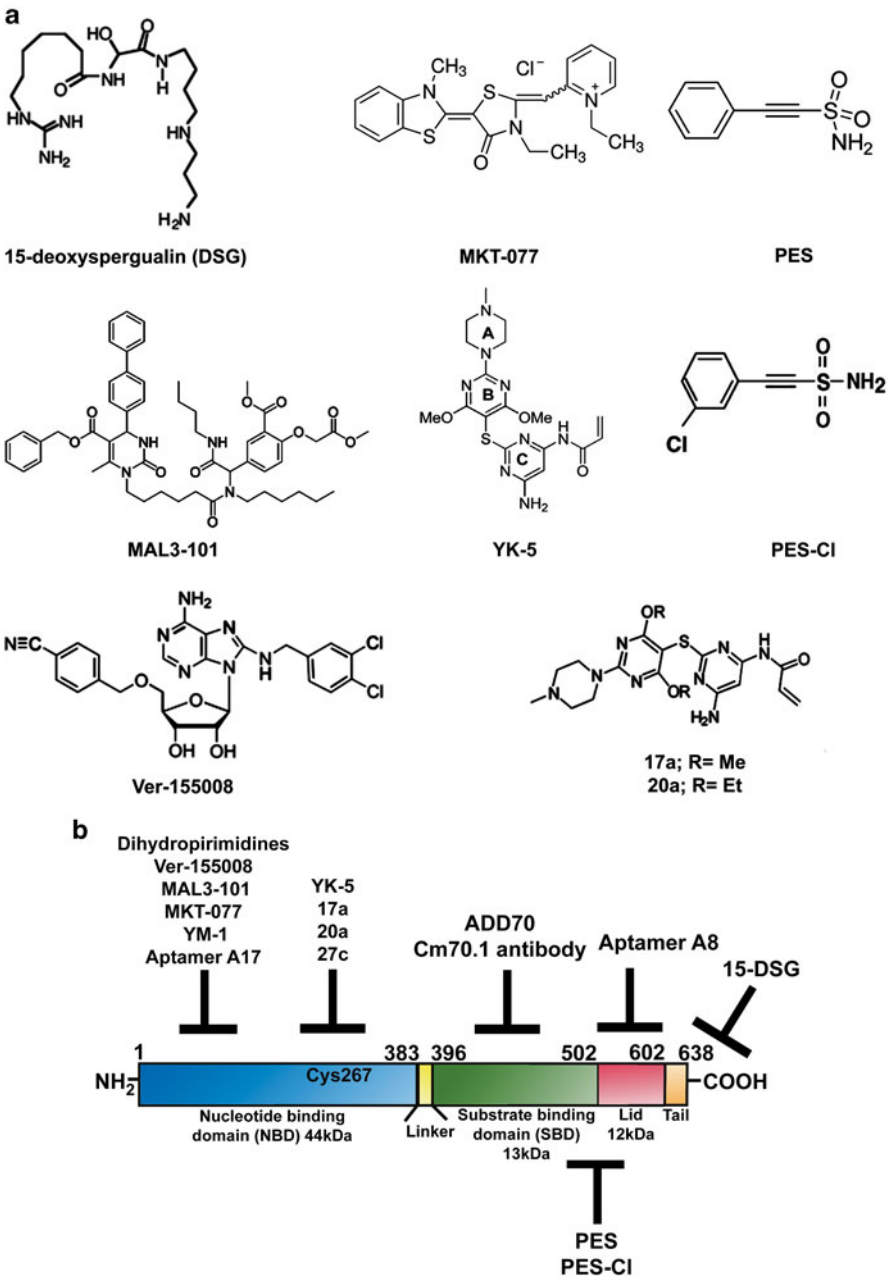

Fig. 10.3 Small molecule inhibitors of HSP70. (**a**) Chemical structures of HSP70 inhibitors. (**b**) Schematic representation of HSP70 domain architecture and the possible sites targeted by the various described inhibitors

interfering with its steady-state ATPase activity (Nadler et al. 1995; Brodsky 1999). Although this compound showed promise as an antitumor agent in a mouse leukemia model (Plowman et al. 1987), its efficacy as an anticancer drug in a clinical trial involving metastatic breast cancer was shown to be limited (Dhingra et al. 1994). A subsequent search for compounds with a structural similarity to DSG led to the identification of a class of dihydropyrimidines that block HSP70 ATPase activity (Fewell et al. 2001, 2004). This second-generation of dihydropyrimidines, called the MAL3 series of compounds, were also developed through a screening process (Fewell et al. 2004). Among them, MAL3-101 deserves special mention. It interferes with the co-chaperone HSP40's ability to stimulate the ATPase activity of HSP70 and prevents its function (Rodina et al. 2007). MAL3-101 blocks cancer cell proliferation (Braunstein et al. 2011) and was shown to be efficacious in a xenograft model of multiple myeloma (Rodina et al. 2007; Braunstein et al. 2011). This series of compounds awaits further testing as prospective anti-cancer drugs.

10.7.2 MKT-077

MKT-077, a rhodocyanine compound, was discovered to accumulate selectively in mitochondria of tumor cells and shown to exert an anti-proliferative effect on cancer cells (Koya et al. 1996). Interestingly, this compound binds an allosteric negatively charged pocket close to the ATP-binding site on HSP70 (Fig. 10.3b), and inhibits the ADP-bound state of this protein, interfering with the HSP70 folding cycle (Rousaki et al. 2011). MKT-077 is primarily toxic to tumor cells and its mechanism of action involves blocking the mitochondrial form of HSP70, HSP70-9 (also called Grp75) (Wadhwa et al. 2000; Rousaki et al. 2011). Mouse xenograft studies demonstrated time- and concentration-dependent antitumor activity for this compound (Chiba et al. 1998). In addition, MKT-077 is cytotoxic to different tumor cell lines, inhibits autophagy and reduces the level of HSP90 client proteins, such as CDK4 and HER-2 (Budina-Kolomets et al. 2014). However, analysis of this compound in a Phase I clinical trial led to severe renal impairment and toxicity in both animal and human subjects, thus limiting further testing and use of this compound (Propper et al. 1999). Recently, a derivative of MKT-077, called YM-1 was developed and shown to be selectively toxic to a variety of tumor cell lines, and to restore tamoxifen sensitivity to tamoxifen-resistant MCF-7 cells (Koren et al. 2012). Thus YM-1 has promising therapeutic potential.

10.7.3 Allosteric Inhibitors of HSP70: Compounds YK-5, 17a, 20a and 27c

Chiosis and colleagues recently identified a previously unrecognized allosteric site in HSP70 using a computational approach (Rodina et al. 2013). Analysis of the geometry and the environment of this computationally-identified allosteric

site provided an initiation point for the development of ligands that would bind to this site. The first allosteric inhibitor designed and tested in this series was YK-5, which was found to block both HSP70 and HSC70 function (Rodina et al. 2011). YK-5 blocks HSP70 function by altering the interactions between HSP70, HSP90 and HOP, thus inhibiting the formation of this chaperone complex. This leads to the destabilization of the HSP90 client proteins HER2, RAF-1 and AKT, thereby leading to their degradation. YK-5 reportedly blocks cell proliferation, induces apoptosis and prevents HSF-1 activation or feedback heat shock response in cancer cells. However, YK-5 was found to be an irreversible covalent modifier of HSP70; therefore, this group further developed this series of inhibitors, leading to the identification of two irreversible derivatives, **17a** and **20a**, which selectively bind HSP70 in cancer cells. Addition of **17a** and **20a** at low micromolar doses led to a reduction in the steady state levels of HSP70-HOP-HSP90 chaperone complex and the degradation of HSP90 client proteins, along with the induction of cell cycle arrest and apoptosis (Rodina et al. 2013; Kang et al. 2014).

10.7.4 Peptide Inhibitors

HSP70 has a prominent cytoprotective role and this protein increases the oncogenic potential of cancer cells and blocks apoptosis by interacting directly with, and neutralizing the function of, apoptotic protease activation factor-1 (APAF-1) (Beere et al. 2000) and apoptosis-inducing factor (AIF) (Ravagnan et al. 2001). Garrido and co-workers utilized this anti-apoptotic characteristic of HSP70 and designed the peptide ADD70 (AIF-Derived Decoy for HSP70), which is both nontoxic and cytosolic, and is able inhibit the interaction of HSP70 with AIF and other client proteins. They employed deletion mutants and computer modeling methods to develop this AIF-derived blocking peptide, corresponding to the amino acids 150–228 of AIF, a region necessary for binding the SBD of HSP70 (Fig. 10.3b) and neutralizing its activity (Schmitt et al. 2003). Further investigation by this group showed that expression of ADD70 peptide in tumor cells decreased their tumorigenicity by increasing infiltration of cytotoxic CD8+ T cells into the tumor. In addition, ADD70 sensitized rat colon cancer cells and mouse melanoma cells to the chemotherapeutic agent cisplatin (Schmitt et al. 2006), indicating that it shows promise as an anti-tumor agent. Due to the large size of the ADD70 peptide, introducing it into tumor cells can be sometimes challenging. To eliminate this limiting factor this group developed smaller peptide aptamers, A8 and A17, which bind to the peptide-binding and the ATP-binding domains of HSP70, respectively, and specifically inhibit the chaperone activity of HSP70. A 13-amino acid peptide was further synthesized from the variable region of A17 (called P17) that specifically blocked HSP70 and induced regression of subcutaneous tumors in vivo, via the recruitment of macrophages and T lymphocytes into the tumor bed (Rerole et al. 2011).

10.7.5 Ver-155008

Massey and co-workers developed the first adenosine-derived inhibitor of HSP70 by designing ATP-analogs that bind the ATP-binding pocket of the chaperone by using the crystal structure of HSC70/BAG-1. This led to the development of the ATP-analog Ver-155008, which binds to HSP70 with a K_D of 0.3 μM and inhibits its activity (Williamson et al. 2009). Ver-155008 is exclusively cytotoxic to cancer cells and downregulates expression of the HSP90 client proteins CDK4, HER2 and RAF-1 in tumor cells (Massey et al. 2010; Budina-Kolomets et al. 2014). It also has anti-proliferative function in a variety of cancer cells; however its ability to induce apoptotic cell death in cancer cells is somewhat limited (Massey et al. 2010). It remains to be seen if derivatives of Ver-155008 can be developed to increase the efficacy of this compound.

10.7.6 The HSP70 Antibody Cm70.1

HSP70 was first reported by Multhoff and co-workers to be localized in the plasma membrane of tumor cells (Multhoff et al. 1995; Multhoff and Hightower 1996), where it is integrated into lipid rafts (Schilling et al. 2009). Although HSP70 lacks a transmembrane domain, its presence in the plasma membrane is believed to be necessary for the stabilization of tumor cell membranes from damage due to environmental stress. Intriguingly, only tumor cells, but not corresponding normal cells, express HSP70 on the surface and stain positively for the IgG1 mouse monoclonal antibody (mAb) cmHsp70.1 (Multhoff and Hightower 2011). The Cm70.1 antibody specifically recognizes an epitope in the C-terminus of the inducible HSP70 (Fig. 10.3b) in viable tumors of both humans in vitro and in mouse tumors in vivo (Stangl et al. 2011). Extensive screening studies in nearly 1,000 primary human tumor biopsies and the corresponding normal tissues indicate that HSP70 is often expressed on the plasma membrane of tumor cells only, and its presence is linked with decreased overall survival of patients; therefore Cm70.1 serves as a negative prognostic marker (Pfister et al. 2007). Interestingly, injecting tumor bearing mice with the cmHsp70.1 antibody significantly inhibited tumor growth and enhanced overall survival, while injection of the peptide corresponding to C-terminal region (aa 450–461) of HSP70 abrogated this effect. This finding suggests that cmHsp70.1 may be a useful therapeutic tool.

10.7.7 PES and Derivatives

2-phenylethynesulfonamide (PES, also known as pifithrin-μ) was initially discovered by Gudkov and colleagues as an inhibitor that blocks the ability of the p53 tumor suppressor protein to traffic to mitochondria, and induce BAX/BAK-dependent apoptosis (Strom et al. 2006). However, at that time, PES was not found

to interact with p53, so the target of this compound, and its activity in cells, was unknown. Leu and colleagues were the first to note that treatment of cells with PES appeared to cause the accumulation of autophagosomes in cancer cells. This group went on to show definitively that treatment with PES caused inhibition of autophagy, a key survival pathway that requires the lysosome, and was considered by some to be the Achilles Heel of cancer cells. Based upon the promise of PES as an autophagy inhibitor, Leu generated biotinylated versions of PES and used these compounds to pull down PES-interacting proteins: the only cellular protein found to interact with PES was HSP70 (Leu et al. 2009). This study thus provided the mechanistic basis for Gudkov's finding that PES inhibits mitochondrial trafficking of p53, as HSP70 was later shown to be required for the ability of proteins to traffic to mitochondria (Pimkina and Murphy 2011).

Leu showed that PES binds the carboxy-terminal substrate-binding domain (amino acids 386–641) of HSP70 and interferes with its protein-folding function. Further investigations showed that PES binds a region located between the substrate binding domain and the C-terminal helical "lid" of this protein (Fig. 10.3b) (Balaburski et al. 2013). Consistent with this finding, exposure of cells to PES prevents binding of HSP70 to critical chaperones namely CHIP and HOP, and inhibits HSP70 function. PES is able to induce cell death in tumor cells through multiple mechanisms: it suppresses macroautophagy (as HSP70 binds to, and stabilizes, the lysosome membrane), inhibits NFκB activation (primarily because IkB-α is an HSP70 target), inhibits function of HSP90 client proteins (Leu et al. 2009, 2011), induces G2/M cell cycle arrest and inhibits the catalytic activity of the anaphase promoting complex/cyclosome (APC/C) in cell-free systems (Balaburski et al. 2013). Interestingly, Budina-Kolomets and colleagues recently compared the activity of three HSP70 inhibitors: Ver-155008, MKT-077 and a more potent derivative of PES, PES-Cl. They concluded that in tumor cells, although all three inhibitors can inhibit autophagy and cause reduced levels of HSP90 client proteins, only PES-Cl can inhibit the APC/C and induce G2/M arrest (Budina-Kolomets et al. 2014). In pre-clinical studies PES (40 mg/kg) and the derivative PES-Cl (20 mg/kg) were shown to protect mice from lymphoma (PES/PES-Cl given once per week for 20 weeks) without any cytotoxic effects to the liver or kidney (Leu et al. 2009; Balaburski et al. 2013). In addition, other groups have demonstrated that PES is effective in killing leukemia cells, without causing cytotoxicity to normal hematopoietic cells, and that this HSP70 inhibitor synergizes with other anti-cancer compounds (Steele et al. 2009; Kaiser et al. 2011).

Recently it was discovered that both HSP70 and HSP90 assist in the stabilization or assembly of the purinosome complex, a dynamic multi-protein complex of enzymes involved in purine synthesis. PES was shown to disrupt the purinosome complex in tumor cells, and this compound is able to do so synergistically with the antimetabolite methotrexate (French et al. 2013). In addition, both HSC70 and HSP70 form the ribosome-associated chaperone system and facilitate co-translational folding and elongation of nascent polypeptides as it emerges from the ribosome exit tunnel. However during various stress-inducing conditions, misfolded proteins sequester the chaperone proteins and interfere with their folding function

and cause translational pausing of early elongating ribosomes. Both PES and VER-155008 are able to recapitulate this condition and cause ribosome pausing including decreased protein translation (Liu et al. 2013).

10.8 Future Perspectives

There is enormous potential for HSP70 inhibition to contribute to cancer therapy. Some barriers to this field exist, however, which must be overcome for the field to move forward. First, the disappointing clinical utility of HSP90 inhibitors has led to some souring of the field on HSP70 inhibitors for cancer therapy. However, one of the reasons that HSP90 inhibitors have poor clinical responses is believed to be because of the substantial upregulation of HSP70 inhibitors in treated cancer cells; this up-regulation is due to the release of the master regulator of heat shock transcriptional response, HSF-1, in response to HSP90 inhibition. One solution to this issue will be to combine HSP90 inhibitors with HSP70 inhibitors; indeed, we have seen that these two inhibitors synergize in the treatment of cancer cells (A. Budina-Kolomets, unpublished data). The second hurdle will be the identification of key "client" proteins for HSP70, and the elucidation of the critical cancer-relevant pathways controlled by this protein. It is now clear that HSP70 protein, like the chaperone HSP90, is subject to extensive post-translational modifications, and the nature and impact of these modifications, and how they may differ in tumor and normal cells, needs to be identified. Finally, because HSP70 undergoes extensive movement associated with ATP/ADP and substrate binding, crystallography of this protein has been challenging: however, the use of NMR and X ray crystallography of domains of this protein is allowing for clearer pictures to emerge. Crossing these hurdles should allow for this promising class of compounds to progress to the clinic.

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Chapter 11
The Ubiquitin-Proteasome System (UPS) as a Cancer Drug Target: Emerging Mechanisms and Therapeutics

Lydia Mata-Cantero, Sofía Lobato-Gil, Fabienne Aillet, Valérie Lang, and Manuel S. Rodriguez

Abstract The Ubiquitin-Proteasome System (UPS) plays an important role in the setting of the cellular response to multiple stress signals. Although the primary function of ubiquitin was initially associated with proteolysis, it is now considered as a key regulator of protein function controlling, among other functions, signalling cascades, transcription, apoptosis or oncogenesis. Failure at any level of the UPS is associated with the development of multiple pathologies including metabolic problems, immune diseases, inflammation and cancer. The successful use of the proteasome inhibitor Bortezomib (Velcade) in the treatment of multiple myeloma (MM) and mantle cell lymphoma (MCL) revealed the potential of the UPS as pharmacological target. Ten years later, new inhibitors tackling not only the proteasome but also different subsets of enzymes which conjugate or de-conjugate ubiquitin or ubiquitin-like molecules, have been developed. Most of them are excellent tools to characterize better the emerging molecular mechanisms regulating distinct critical cellular processes. Some of them have been launched already while many others are still in pre-clinical development. This chapter updates some of the most successful efforts to develop and characterize inhibitors of the UPS which tackle mechanisms involved in cancer. Particular attention has been dedicated to updating the status of the clinical trials of these inhibitors.

Keywords Proteasome • Chemical-inhibitors • Bortezomib • Clinical trials • Cancer • Ubiquitin • SUMO • NEDD8 • Conjugating enzymes • Ligases • Isopeptidases • Deubiquitylating enzymes

L. Mata-Cantero
Inbiomed. Paseo Mikeletegi 81, Parque Tecnológico de Miramón, San Sebastian 20009, Gipuzkoa, Spain

Centro de Investigación Básica (CIB), GlaxoSmithKline, Santiago Grisolía 4, Tres Cantos 28760, Madrid, Spain

S. Lobato-Gil • F. Aillet • V. Lang • M.S. Rodriguez (✉)
Inbiomed. Paseo Mikeletegi 81, Parque Tecnológico de Miramón, San Sebastian 20009, Gipuzkoa, Spain
e-mail: msrodriguez@inbiomed.org

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11.1 Introduction

The ubiquitin-proteasome system (UPS) is the major proteolytic system in the cytosol and nucleus of all eukaryotic cells (reviewed in Weissman et al. 2011). Most of our initial understanding of this pathway comes from biochemical approaches and genetic studies in yeast (reviewed in Weissman et al. 2011). Knowledge about the physiological roles of the UPS in mammalian cells was quite slow until cell-permeable proteasome inhibitors were developed (Palombella et al. 1994; Tsubuki et al. 1993). Proteasome inhibitors helped to demonstrate that 26S and 20S proteasomes contribute to the degradation of most short-lived and long-lived proteins that exhibit critical functions inside the cell such as transcription, apoptosis or cell cycle regulation, either through ubiquitin-dependent or ubiquitin-independent mechanisms (Glass and Gerner 1987; Tanaka et al. 1983). Consequently, UPS deregulation is linked to different human pathologies (Schwartz and Ciechanover 1999). In this chapter, we will focus on UPS inhibitors used in the treatment of cancer.

11.1.1 Ubiquitin Proteasome-System

The UPS is a complex system composed of multiple molecular machineries acting in a synchronous manner to maintain a dynamic equilibrium of their components. The UPS is often represented as two groups of separated molecular mechanisms: the first group comprises specific enzymes and cofactors acting to modify/de-modify protein substrates with a single member of the ubiquitin family; the second group of the UPS is the 26S proteasome, of approximately 2,000 kDa, which is responsible for the proteolysis of labelled substrates.

11.1.2 Protein Modification by Ubiquitin and Ubiquitin Family Members

The ubiquitin family of protein modifiers, also known as Ubiquitin-like modifiers (UbLs), is composed of at least 15 members sharing a modest primary sequence identity with ubiquitin, but preserving its compact globular β-grasp fold (Hochstrasser 2009). In mammalians, the family includes the small ubiquitin-like modifier (SUMO), the neuronal precursor cell-expressed, developmentally down-regulated protein-8 (NEDD8, also known as Rub1 in yeast), the ubiquitin cross-reactive protein (UCRP, alternatively named interferon-stimulated gene-15 ISG15), the ubiquitin-related modifier-1 (URM1), the human leukocyte antigen F-associated (FAT10), the Fau ubiquitin-like protein (FUB1), the MUB (membrane-anchored UBL), the ubiquitin fold-modifier-1 (UFM1), the ubiquitin-like protein-5 (UBL5, homologous to ubiquitin-1 [Hub1] in yeast), and the autophagy proteins ATG8 and ATG12.

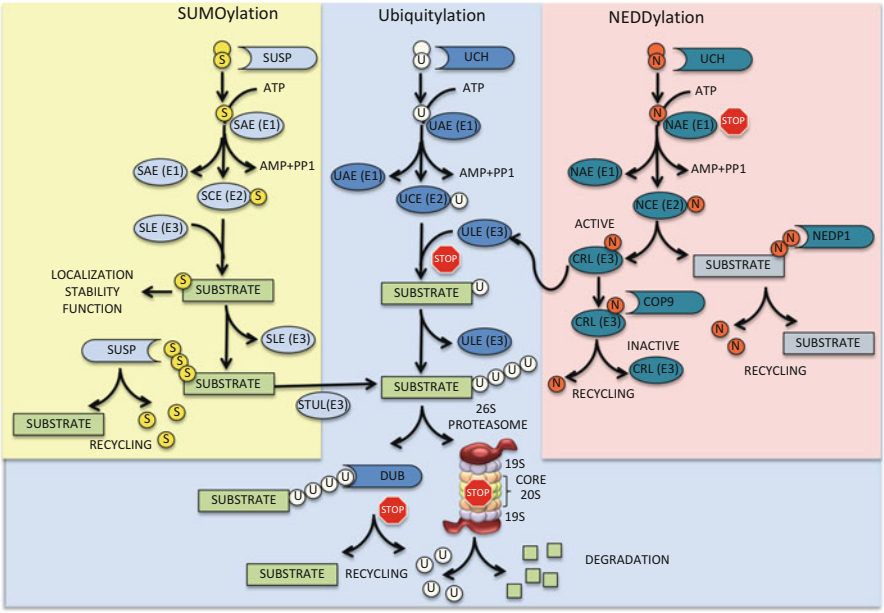

Fig. 11.1 Available drugs in clinical trials to block the Ubiquitin-Proteasome System. All members of the ubiquitin family are generated through a high molecular weight precursor that has to be cleaved by specific proteases to generate the mature form of each modifier. The covalent attachment of ubiquitin is mediated by a thiol-ester cascade of reactions involving at least three enzymes: an activating enzyme (AE) or E1; a conjugating enzyme (CE) or E2; and a ligase (LE) or E3. Ubiquitin-like proteins such as SUMO or NEDD8 have their own set of enzymes. Demodifying enzymes such as DUBs, SUSPs or NEDP1 participate in a proofreading mechanism. Multiple chain types can be formed and the composition of the chains is essential to drive distinct functions, including proteolysis by the 26S proteasome

Ubiquitin and all members of the ubiquitin family are attached to protein substrates through biochemical processes which are similar but implicate distinct sets of enzymes with the capacity to act on a limited number of reactions (Fig. 11.1). All protein modifiers of the ubiquitin family are generated through the proteolytical cleavage of higher molecular weight precursors that exposes the double glycine (GG) signature, typically found in this family, and which is required for their attachment to protein substrates (reviewed in Jentsch and Pyrowolakis 2000). The attachment (or conjugation) of ubiquitin or UbL modifiers is mediated by a thiol-ester cascade of reactions that requires the action of three enzymes: an activating enzyme or E1 that will activate all molecules required for all reactions and a conjugating enzyme or E2 that, in most cases, will conjugate protein modifiers to their targets. Ubiquitin ligases or E3s are responsible for, or are required to achieve the conjugation of the modifier to its target protein (Fig. 11.1). The role that an E3 will exert depends on its capacity to form thiol-ester intermediates with protein modifiers.

The ubiquitin ligases of the HECT family are typical examples of these active enzymes. Another category of E3 ligases contributes to bring together substrates and E2s in order to achieve protein conjugation without having any enzymatic activity. Among this category of E3s, we found the cullin-RING ligase (CRLs) composed of multiple subunits that will allow the specific recognition of a degradable substrate (e.g. after its phosphorylation). The contribution of E4 factors favouring chain extension has also been proposed (Hoppe 2005). Chain formation is essential to drive distinct functions. While ubiquitin chain linkages K48 and K11 have been associated with degradation, K63 chains appear to connect signalling cascades, endocytic trafficking or DNA repair. The formation of hybrid chains containing ubiquitin and UbLs such as SUMO2/3 is also possible (Aillet et al. 2012; Guzzo and Matunis 2013; Kulathu and Komander 2012) but a generic function of these chains has not been established. Deubiquitylating enzymes or DUBs recognize specific ubiquitin chains from target substrates through ubiquitin binding domains (UBDs) to know which chains should be cleaved (Eletr and Wilkinson 2014). An equivalent reaction is achieved by SUMO-specific proteases (SUSPs) or NEDD8 specific protease (NEDP1), also known as Denedylase 1 (DEN1) (Hickey et al. 2012). All isopeptidases show specificity for a modifier, however due to saturated in vitro assays, overexpression or certain stress conditions, cross-reactions have been reported thus making any conclusion on the exclusivity of these reactions difficult. Although the initial drug discovery strategies used enzymes recognizing protein substrates (E3s and isopeptidases) to develop new chemical inhibitors, nowadays all enzymes are considered as good candidates for drug development.

11.1.3 Proteasomes

The 26S proteasome is a multi-subunit complex formed by a barrel-shaped proteolytic core, the 20S core particle, and one or two regulatory 19S particles flanking the ends of the core to regulate the entry of proteins targeted for degradation (Fig. 11.1) (Bedford et al. 2010). The 20S core particles consist of four stacked heptameric ring structures that are themselves composed of two different types of subunits. The two outer rings are formed by seven alpha subunits (α1–α7) that allow the interaction with the 19S particle, while the inner rings are composed of seven beta subunits (β1–β7), three of which are responsible for the catalytic activity. The catalytic mechanism of these subunits is the same because they use the hydroxyl group of the N-terminal threonine of a mature β-subunit to perform the nucleophilic attack on the carbonyl carbon of a peptide bond (Borissenko and Groll 2007; Kisselev and Goldberg 2001; Kisselev et al. 2012; Voges et al. 1999). Each subunit reacts with specific substrates: β1 subunit possesses peptidyl-glutamyl peptide-hydrolyzing (PHGH) activity that cleaves after acid residues, β2 has trypsin-like activity that cleaves after basic residues and β5 subunit shows chymotrypsin-like activity that

cleaves after hydrophobic residues. Site-directed mutagenesis studies of these catalytic residues in yeast showed that the most drastic changes occurred when β5 subunit is inactivated (Heinemeyer et al. 1997).

The 19S is divided into two sub-particles, the base and the lid. The lid contains at least nine non-ATPase polypeptide chains (Rpn3, 5–9, 11, 12 and 15) that remove the polyubiquitin chains from the protein-substrates, while the base consists of four non-ATPase (Rpn1, Rpn2, and Rpn13) and six ATPase subunits (Rpt1–Rpt6) that interact directly with α-rings. These ATPases control the opening of the 20S gate that is normally locked until an unfolded substrate is recognized. Only the unfolding step involves ATP hydrolysis (Peth et al. 2010).

Apart from the proteasome, an immuno-proteasome exists in immune cells where the 11S subunit, also known as PA28 or REG, replaces the 19S subunit. The 11S is dominantly expressed in hematopoietic cells in response to pro-inflammatory signals such as interferon gamma or cytokines, and is involved in antigen processing for subsequent presentation to the MHC-I on the cell surface, allowing the initiation of the immune cell response (Rock et al. 1994; Tanaka and Kasahara 1998). Furthermore, the immune-proteasome facilitates the clearance of protein aggregates to prevent cell death produced by IFN-induced oxidative stress, (Seifert et al. 2010). All proteasome inhibitors currently used in clinical trials block the enzymatic activities of the proteasome. However, the next generation of proteasome inhibitors will block other mechanism such as gate opening or regulatory subunits.

11.2 Targeting Proteasomes

The first proteasome inhibitors made available were synthesized to specifically block the proteasome's active sites and to understand its enzymatic mechanism. Peptide aldehydes such as MG-132 were used to develop analogues with enhanced potency, selectivity and stability. Surprisingly, several studies showed that proteasome inhibitors induced rapid and selective apoptosis in different cancer derived cell lines, leading to the idea that proteasome inhibitors could be drug candidates. This idea was confirmed by the fact that the proteasome inhibitor Bortezomib regressed tumour size of xenograft tumours and also decreased metastasis and blocked angiogenesis in patients with hematologic malignancies. Thus, Bortezomib was the first proteasome inhibitor approved by the Food and drug Association of the United States (FDA). Since its approval in 2003, it became the frontline treatment for MM, it has been accepted as second line treatment of MCL and it has also been included in hundreds of on-going clinical trials. In addition to Bortezomib, Carfilzomib, another proteasome inhibitor, has been approved for relapsed/refractory MM, and other proteasome inhibitors are in clinical and preclinical trials (Table 11.1).

Table 11.1 Proteasome inhibitors in clinical trials

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Disease	Ref
Bortezomib (Velcade)	Takeda	Proteasome β5 > β1> β2	Boronate	Reversible	IV SC IP	Launched	Cancer: Multiple myeloma, non-Hodgkin's lymphoma	Adams et al. (1998) McBride and Ryan (2013) Frankland-Searby and Bhaumik (2012) www.clinicaltrials.gov
						Phase II	Cancer: B-cell lymphoma, Hodking's lymphoma, leukaemia chronic lymphocytic, leukaemia acute myelogenous, small cell lung, non-small cell lung, prostate, gastrointestinal, breast, renal, liver, head and neck, ovarian, colorectal, oesophageal, pancreatic, sarcoma, bladder, glioblastoma, tyroid, osteosarcoma, mesothelioma, myelodyplastic syndrome, waldenstrom macroglobulinemia, unspecific solid tumor	
							Others: Lupus, HCV, HIV, cytomegalovirus infections, thrombotic disorders	
						Phase I	Cancer: Melanoma, testicular, endometrial, metastatic, medulloblastoma	

Carfilzomib (PR171)	Onyx	Proteasome β5 >> β1, β2	Epoxyketone	Irreversible	IV	Launched	Cancer: Multiple Myeloma	Frankland-Searby and Bhaumik (2012) McBride and Ryan (2013) Demo et al. (2007) Xolalpa et al. (2013) Pautasso et al. (2013) www.clinicaltrials.gov
						Phase II	Cancer: Non-Hodgkin's lymphoma, leukaemia chronic lymphocytic, small cell lung, non-small cell lung, renal, unspecified solid	
							Others: Cytomegalovirus infections	
						Phase I	Cancer: Hodgkin's lymphoma, leukaemia acute lymphocytic, leukaemia acute myelogenous	
MLN9708 (Ixazomib)	Takeda	Proteasome β5 > β1 > β2	Boronate	Reversible	IV Oral	Phase III	Cancer: Multiple myeloma	Frankland-Searby and Bhaumik (2012) McBride and Ryan (2013) Kupperman et al. (2010) Xolalpa et al. (2013) www.clinicaltrials.gov
						Phase II	Cancer: Non-Hodgkin's lymphoma, leukemia acute myelogenous	
						Phase I	Cancer: Hodgkin's lymphoma, leukemia acute lymphocytic, leukemia chronic lymphocytic, waldenstrom's hypergam-maglobulinaemia, non-small cell lung, colorectal, melanoma, renal, sarcoma, prostate, pancreatic, head and neck, endometrial, breast, gastric, esophageal, testicular, liver, thyroid, unspecified solid	

(continued)

Table 11.1 (continued)

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Disease	Ref
CEP-18770 (delanzomib)	Teva	Proteasome β5 > β1 > β2	Boronate	Reversible	IV Oral	Phase II	Cancer: Multiple myeloma	Xolalpa et al. (2013) Piva et al. (2008) Frankland-Searby and Bhaumik (2012) www.clinicaltrials.gov
						Phase I	Cancer: Non-Hodgkin's lymphoma, non-small cell lung, colorectal, head and neck, ovarian, renal, prostate, unspecified solid	
ONX-0912 (oprozomib)	Onix	Proteasome β5	Epoxyketone	Irreversible	Oral	Phase II	Cancer: multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia chronic lymphocytic, leukemia chronic myelogenous, waldenstrom's hypergammaglobulinaemia	Xolalpa et al. (2013) Frankland-Searby and Bhaumik (2012) Chauhan et al. (2010) www.clinicaltrials.gov
						Phase I	Cancer: non-small cell lung, liver, prostate melanoma, ovarian, renal, gastric, colonorectal, medulloblastoma, breast, sarcoma, unspecified solid	
NPI-0052 (marizomib)	Nereus	Proteasome β5 > β2> β1	B-lactone	Irreversible	IV Oral	Phase I	Cancer: Multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukaemia chronic lymphocytic, leukaemia acute lymphocytic, leukaemia acute myelogenous, non-small cell lung, sarcoma, pancreatic, melanoma, prostate, liver, ovarian, breast, renal, gastric, unspecified solid	Xolalpa et al. (2013) Fenical et al. (2009) McBride and Ryan (2013) Frankland-Searby and Bhaumik (2012) www.clinicaltrials.gov

Syrbactins, Pono Pharma	Pono Pharma	Proteasome	Syrbactin	Irreversible	Unspecified	Preclinical	Cancer: Myeloma, unspecified	Groll et al. (2008)
Proteasome inhibitor IkerChem	IkerChem	Proteasome	Unspecified	Unspecified	Unspecified	Preclinical	Cancer: Haematological unspecified, solid unspecified	http://www.ikerchem.com/our_research.html
Peptide epoxyketones Onyx Pharmaceuticals	Onyx Pharmaceuticals	Proteasome	Epoxyketone	Unspecified	Unspecified	Preclinical	Immunological: Inflammatory disease unspecified	http://www.onyx-pharm.com/
ONX-0914 (PR-957)	Onyx Pharmaceuticals	Inmunoproteasome β5i/LMP7	Epoxyketone	Irreversible	Unspecified	Preclinical	Immunological: Arthritis, rheumatoid, psoriasis, inflammatory bowel disease, lupus erythematosus Cancer: haematological, unspecified	Verbrugge et al. (2012)
FV-162	Fluorinov Pharma	Proteasome β5	Fluorine	Irreversible	Oral	Preclinical (Phase I planned)	Cancer: Myeloma, non-Hodgkin's lymphoma	http://www.fluorinovpharma.com/
Bortezomib micelle	NanoCarrier	Proteasome β5 > β1> β2	Boronate	Reversible	Unspecified	Preclinical	Cancer: unspecified	http://www.nanocarrier.co.jp/en/research/pipeline/index.html
Timosaponin AIII (BN-108)	Bionovo	Proteasome	Steroidal saponin	Unspecified	Oral	Preclinical (Phase I/II planned)	Cancer: breast	Chun-Nam et al. (2011)

11.2.1 Proteasome Inhibitors in Clinical Use

The new generation of proteasome inhibitors always aims to increase potency, specificity and stability with good bioavailability and pharmacokinetics. Conventional proteasome inhibitors efficiently block at least one of the three proteasome active sites (β1, β2 and β5) either with covalent or non-covalent, reversible or irreversible binding. Most Inhibitors and drugs in clinical trials are mimetic peptides that interact directly with the active site thus blocking the nucleophilic attack of the hydroxyl group of the proteasome N-terminal threonine active sites (Kisselev and Goldberg 2001; Orlowski and Kuhn 2008). Proteasome inhibitors are often classified according to their functional group (Table 11.1):

Boronic acid peptides: Peptide aldehyde analogues were synthetized substituting the aldehyde group for boronic acid (**Bortezomib**) (Adams et al. 1998). Bortezomib is more potent, stable and interacts more specifically with the β5 subunit, forming tetrahedral intermediates with two extra hydrogen bonds that stabilize the covalent bond (Groll et al. 2006). Bortezomib is a reversible inhibitor administrated intravenously with very low dissociation rate that behaves as an irreversible molecule. It has a dose limiting toxicity and produces multiple side effects in patients, including pain, fatigue, gastrointestinal, cardiovascular and pulmonary disorders, neutropenia, thrombocytopenia and peripheral neuropathy. Despite its potency and effectiveness, about 60 % of the treated patients develop resistance to Bortezomib after 1 year of treatment (Mujtaba et al. 2012). The resistance mechanisms are still poorly understood but have a multifactorial basis (Xolalpa et al. 2013).

A second-generation of proteasome inhibitors, currently in clinical trials, include the boronic acid peptides **MLN2238**, **MLN9708** and **CEP-18770**. Unlike Bortezomib, these inhibitors reduce neuropathy. MLN2238 and its oral analogue MLN9708, are reversible inhibitors in phase III trials with stronger chymotrypsin-like activity inhibition in vivo and a faster dissociation rate, able to penetrate inside tissues (Kupperman et al. 2010). The oral inhibitor CEP-18770 in phase I and II trials shows encouraging results for the treatment of haematologic and solid tumours (Piva et al. 2008).

Epoxyketone peptides. These peptides are the most potent and specific proteasome inhibitors known. They form a morpholine adduct with the N-terminal threonine through a covalent and an irreversible bond (Groll et al. 2000). **Carfilzomib** (Demo et al. 2007) and **ONX-0912** (Chauhan et al. 2010) belong to this class of inhibitors. They show a high chemical stability causing a stronger inhibition of the chymotrypsin-like proteasome activity than Bortezomib (Huber and Groll 2012), and overcome the problem of resistance to Bortezomib. Due to their higher specificity, neurotoxicity is reduced (Molineaux 2012) but other side effects still persist (Fostier et al. 2012). Carfilzomib is currently used for relapsed and refractory multiple myeloma and the orally bioavailable ONX-0912 is included in phase I and II trials where it has shown an improved therapeutic window.

β-***lactones***: *Non-peptidic inhibitors* have also been developed to improve bioavailability. The compound derived from the marine microorganism *Salinispora*

tropica, **Marizomib**, is a β-lactone inhibitor in phase I trials (Fenical et al. 2009). Its β-lactone group reacts with the catalytic threonines of the proteasome active site forming an acyl-ester adduct and a tetrahydrofuran ring that stabilizes the binding leading to a highly potent, selective and orally bioactive proteasome inhibitor. Contrary to epoxyketones and peptide boronic acids, Marizomib inhibits the three activities of the proteasome irreversibly, in a stronger (more than 90 %) and longer-lasting way (Miller et al. 2011). The use of marizomib results in adverse effects such as fatigue, nausea, vomiting and dyspnea.

11.2.2 Development of New Proteasome Inhibitors

A second generation of proteasome inhibitors is being designed to specifically inhibit a subunit of interest, to reduce toxicity in normal cells and increase apoptosis in tumour cells (Parlati et al. 2009). This is the case of **FV-162** that shows a better inhibition of chymotrypsin-like with an improved safety profile compared to carfilzomib, and the epoxyketone **ONX 0914**. ONX 0914 is a potent drug orally bioavailable for the immunoproteasome and has been developed for the treatment of autoimmune disorders.

Moreover, natural products and non-covalent proteasome inhibitors are also being developed to reduce side effects. **Timosaponin AIII** is an example of a natural product in preclinical development whose phases I/II in breast cancer are under preparation. **Withaferin A** and **gambonic acid** are other natural inhibitors, which have been approved by the Chinese FDA for cancer clinical trials.

To avoid resistance to proteasome inhibitors, and to limit the off-target effects, the development of compounds acting in a non-competitive way that do not directly interact with the active catalytic β subunit's site, has been considered. These compounds could potentially be used in combination with Bortezomib to improve clinical outcomes. For example, **Rapamicin** and **PR-39** are compounds that bind to the α7 subunits, thereby producing a disruption in the interaction between 20S and 19S regulatory subunits that blocks the entry of the substrate (Gaczynska et al. 2003). **5-amino-8-hydroxyquinoline** also interacts with the α-subunits inside the proteolytic chamber; while being cytotoxic for myeloma and leukemia cells in vitro, it has also been shown to decrease tumour size in xenograft tumour growth in vivo (Li et al. 2010), and overcomes resistance to Bortezomib in cultured cell lines.

11.2.3 Cellular Effects of Proteasome Inhibitors

Proteasome inhibitors are successful in cancer cells with high proliferative rates. Although all mechanisms are not fully understood, it seems that they block the degradation of proapoptotic proteins and cell cycle regulators (Fig. 11.2). Among them, the tumor suppressors retinoblastome (Rb), p53 and the cyclin-dependent kinase inhibitors (CDKIs), such as p21Cip1, p27Kip1 and p57Kip2 are degraded by the proteasome (Frankland-Searby and Bhaumik 2012; Rastogi and Mishra 2012; Vlachostergios et al.

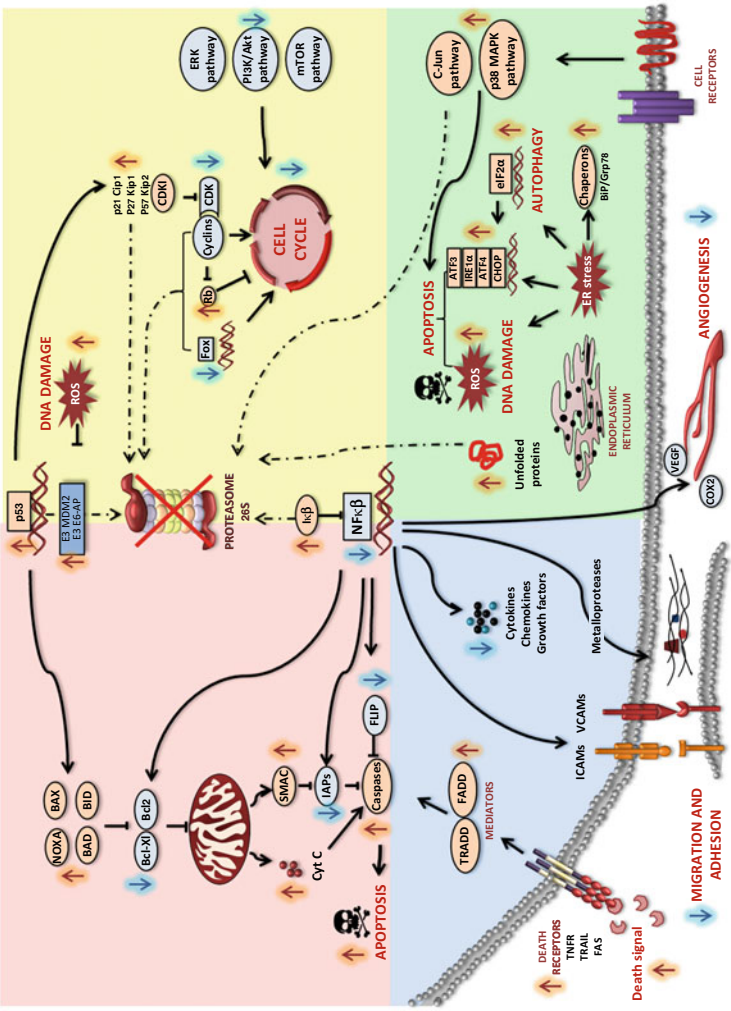

Fig. 11.2 Cellular effects of proteasome inhibition. Inhibition of the proteasome stabilizes and activates critical factors that control several apoptotic pathways in cancerous cells. Most pathways affected are involved in cell cycle control, cell proliferation, oncogenic activity, apoptosis, tumour suppressor functions and includes other multiple regulatory proteins (see main text)

2013). Regulators of the intrinsic apoptotic pathway are also controlled by the proteasome, including members of the Bcl-2 protein family (p53-target-genes) such as the proapoptotic proteins Bax, Bid, Noxa or Bad, and anti-apoptotic members such as Bcl-XL and Bcl-2. Bortezomib treatment increases the expression of proapoptotic members such as Bax, Smac and Noxa while anti-apoptotic proteins like Bcl-2 and IAPs are down-regulated, thus favoring apoptosis in tumor cells (Crawford and Nahta 2011).

The mechanism to induce apoptosis of each proteasome inhibitor seems to be different, thus some combinations result in synergies. For example, Carfilzomib and Marizomib induce apoptosis mediated by caspase 8 and 9 better than Bortezomib, which is more dependent on Bax and Bak mitochondrion-mediated cell death (Kuhn et al. 2007; Miller et al. 2007).

The inhibition of NFκB by Bortezomib seems to have an important role in this drug's mechanism of action, although it cannot explain all the anticancer effects (Fig. 11.2). NF-κB-mediated transcription plays a crucial role in tumorigenesis by controlling among others the immune and inflammatory responses; apoptosis suppression (by inducing anti-apoptotic IAPs and BCL-XL proteins); angiogenesis promotion (by inducing vascular endothelial growth factor VEGF and COX2); favouring cell proliferation (by increasing cyclin D) and increasing cell migration (by inducing metallo-proteases) (Roccaro et al. 2006). Furthermore, constitutive expression of NF-κB has been related with resistance against radiation and chemotherapy in various types of cancer (Sunwoo et al. 2001).

Disruption of the proteasome activity also leads to the aggregation of unfolded proteins, thereby producing ER stress. An extensive protein production and secretion has been found in different types of cancer, becoming more sensitive to ER stress (Hoeller and Dikic 2009). ER stress leads to up-regulation of the endoribonuclease/kinase IRE1a and the transcription factors ATF3&4 that will increase transcriptional activation of the pro-apoptotic Noxa (Armstrong et al. 2010). Autophagy is also induced by ER stress as a resistance mechanism to escape cell death through the eIF2α induced pathway, the activation of HDAC6, the IRE1-JNK pathway, by proteasomal stabilization of ATF4, the inhibition of mTOR, and by the reduced proteasomal degradation of LC3 (Belloni et al. 2010). The activation of p38 MAPK and c-Jun N-terminal kinases has been also reported after proteasome inhibition. This leads to 14-3-3 phosphorylation and consequently to cytochrome c release. The generation of reactive oxygen species (ROS) after the treatment with proteasome inhibitors results in the expression of apoptosis-related proteins (Fan et al. 2011). ROS are responsible for DNA damage and proteasome plays a crucial role in DNA repair through the activation of p53 and other pathways.

11.2.4 Experience with Clinical Trials

Bortezomib has shown promising activity and a durable response as a single agent in relapsed or refractory MCL and MM patients (Table 11.1). Trials in Phase I demonstrated an effective plateau at 65–70 % proteasome inhibition with fair

tolerance levels. In phase II, 35 % of patients with refractory MM and 46.5 % of patients with pre-treated MCL responded to Bortezomib, therefore the FDA initially approved it for relapsed/refractory MM in 2003, and subsequently for relapsed/refractory MCL in 2006 (Kane et al. 2006). Moreover, survival rates of refractory MM patients using Bortezomib in phase III trials exceeded the response rates for patients treated with the previous drug choice Dexamethasone, resulting in Bortezomib being approved as a first-line therapy in 2008 (Richardson et al. 2005). However the refractory response to Bortezomib depends on the tumour cell type. Pancreatic, prostate and non-small lung tumour cells are sensitive while diffuse large B cell lymphoma, follicular lymphoma, Waldenstrom's macroglobulinemia and some solid tumours such as sarcoma, renal cell carcinoma and glioma only provide slight and short responses (Frankland-Searby and Bhaumik 2012).

To overcome Bortezomib resistance, numerous combinations with chemotherapeutic agents, immunomodulators and/or other proteasome inhibitors are under investigation. In 2007, the FDA approved the use of a Bortezomib/Doxorubicin combination since it was better than the use of single compounds in phase III trials. Combinations with Doxorubicin/Adriamycin (ADR), Thalidomide, Melphalan, Dexamethasone, Cyclophosphamide and Myriad have also been successful. The most active combination to date includes Bortezomib, Lenalidomide and Dexamethasone, where overall responses of 100 % were obtained in phase II. Early phase I and II clinical trials using combinations of Bortezomib with new immunomodulators, radio-immunotherapy, stem cell transplantation, monoclonal antibodies or chemotherapeutic agents have been promising. In addition, targeted therapies such as histone deacetylase inhibitors, Bcl-2 inhibitors, rapamycin inhibitors, multiple kinase inhibitors (Akt, PKC, CDK inhibitors, etc.) and heat shock protein inhibitors simultaneously used with Bortezomib also show encouraging results (McBride and Ryan 2013).

MLN9708 shows synergistic effects in association with Bortezomib, vorinostat, lenalodomide and dexamethasone, in refractory MM (phase III) and first line MM patients (phase I/II) (McBride and Ryan 2013). Studies in phase I/II with **Carfilzomib** exhibit durable responses with acceptable toxicity in relapsed/refractory MM patients compared to Bortezomib and stem cell transplants, achieving a 24 % partial response in heavily pre-treated patients (McBride and Ryan 2013; Pautasso et al. 2013). Several phase II trials are currently on-going for haematological malignancies and solid tumours. Phase I/II trials with different compound combinations including Calfizomib/Thalidomide/Dexamethasone/Cyclosporamide are being also tested as a frontline treatment for MM, showing promising activity with high response rates in the first patients evaluated.

The last proteasome inhibitor that entered into phase I clinical trials was **Marizomib**, used alone or in combination with Dexamethasone for the treatment of different kinds of cancer (see Table 11.1). In preclinical trials, Marizomib is also combined with Bortezomib, since different mechanisms of action have been suggested (McBride and Ryan 2013).

11.3 Targeting NEDDylation

Together with ubiquitin and SUMO, NEDD8 is among the most studied ubiquitin-like proteins (Fig. 11.1). The best known physiological substrates of NEDDylation are the cullins, which are structurally related proteins that function as molecular scaffolds of the cullin-RING family of E3 ubiquitin-ligases (CRLs). CRLs consist of a core cullin protein bound to a RING finger protein (Rbx1/2) and an interchangeable substrate-binding adaptor protein (Bedford et al. 2011; Watson et al. 2011). Rbx1 and Rbx2 in conjunction with E2 enzymes UBC12 and UBE2F, respectively, promote NEDDylation of cullins (Huang et al. 2009). The archetypal CRLs, the so-called SCF ubiquitin ligases, contain CUL1, RBX1/2 and the adaptor protein Skp1. This core complex binds to one of the approximately 100 F-box proteins that are responsible for recruiting substrates (Bedford et al. 2011). Cullin NEDDylation has been shown to increase the ubiquitylation activity of CRLs by promoting conformational changes that increase the binding of Rbx1 to ubiquitin E2s, which results in a reduction of the distance between E2 and the substrate recognition component; it may also lead to displacement of the negative regulatory protein, CAND1, that binds to non-NEDDylated cullin (Watson et al. 2011). Specifically, the CRLs have been established to control the degradation of proteins with important biological roles, including cell cycle progression (p27, cyclin E, c-Myc), tumour suppression (p53), DNA damage (CDT1), stress responses (NRF-2, HIF-1α), and signal transduction (IκBα). In addition, other NEDD8-regulated substrates with key cancer-related functions are β-catenin, c-JUN, mTOR and MDM2. Interestingly, increasing evidence suggests that NEDD8-mediated protein turnover may be deregulated in malignant cells and could result in oncogenic transformation, disease progression, or impart a drug-resistant phenotype (Nawrocki et al. 2012). For example, disruptions in the NEDD8 pathway lead breast cancer cells to acquire anti-estrogen resistance and expression of ER alpha (Fan et al. 2003). It has also been shown that increased NEDD8 conjugation appeared to be essential for the enhancement of proliferation in several types of human carcinoma cells (Chairatvit and Ngamkitidechakul 2007).

11.3.1 NEDDylation Inhibitors

Because many CRL substrates have tumour suppressor activity, preventing the degradation of these proteins could be an effective anticancer strategy that may also help to reduce toxicities resulting from global proteasomal inhibition (Nawrocki et al. 2012). **MLN4924**, a potent and selective first-in-class small molecule inhibitor developed by Millennium Pharmaceuticals, was reported as a specific inhibitor of protein NEDDylation through the inactivation of the heterodimer APPBP1 (NAE1) and UBA3 (UBE1C), also known as the NEDD8 activating enzyme NAE (Soucy et al. 2009; Wang et al. 2011). MLN4924 is an adenosine sulphamate that forms a

covalent adduct with NEDD8 when bound to the NAE active site and in this way inhibits the formation of UBC12-NEDD8 thioester. The MLN4924-NEDD8 adduct *mimics* the NEDD8-AMP in situ which is the first intermediate in the NAE reaction cycle, but cannot be enzymatically processed further (Brownell et al. 2010; Nawrocki et al. 2012). MLN4924 inhibits CRL activity and the stabilization of their substrates by blocking NAE, with subsequent implications in cancer cell growth and survival (Bedford et al. 2011). In 2009, MLN4924 was first reported as a potent growth suppressor agent against a variety of cancer cells lines derived from solid tumours and haematological malignancies in both in vitro and in vivo xenograft models (Soucy et al. 2009; Zhao et al. 2014). During preclinical trials, tumour regression occurs after the action of different mechanisms depending on context and cell type. Induction of apoptosis is one of the reported effects mediated by MLN4924 in three different mechanisms mainly caused by the accumulation and stabilization of CRL substrates like: (1) CDT1, which trigger DNA re-replication and S phase arrest; (2) IκBα, that blocks NF-κB activation, and; (3) pro-apoptotic NOXA. In addition to apoptosis, MLN4924 also induces autophagy in a concentration- and time-dependent manner in multiple human cancer cell lines derived from carcinomas of breast, colon, liver, brain and cervix. Studies revealed that autophagy is mainly caused by inactivation of mTORC1, most likely mediated by accumulation of DEPTOR and HIF1α, followed by the activation of the HIF1-REDD1-TSC1 axis. Some studies have also shown that MLN4924 can induce irreversible senescence in multiple cancer cell lines in a p21-dependent manner. In SK-BR3 cells, p16 and p27 accumulation may also contribute to the MLN4924-induced senescence. Furthermore, MLN4924 has a potential sensitizer role in chemotherapy and radiation, involving the mechanistic accumulation of c-Jun, NOXA, CDT1, WEE1 or p21; it also affects the promotion of c-FLIP degradation, increasing the expression of Bcl-2-interacting killer (BIK), inactivating CRL3, as well as the suppression of FANCD2 monoubiquitylation and CHK1 phosphorylation, all of which results in a general increase in cellular sensitivity by enhancing DNA damage, oxidative stress, cell cycle arrest and finally apoptosis (Zhao et al. 2014). Despite this promising optimal strategy to inhibit NAE pathway, recently it has been identified that cancer cells can develop resistance to MLN4924 treatment. Resistance has been linked to heterozygous mutations in two areas of NAEβ (UBA3), the ATP binding pocket at Alanine 171 and at various residues within or close to the NEDD8-binding cleft. A point mutation in residue A171T reduced affinity for both MLN4924 and ATP. Interestingly, such resistance has been effectively bypassed by using a compound with tighter binding properties for NAE. These findings provide critical clinical aspects with respect to patient selection and consolidate efforts for the development of next-generation NAE inhibitors in order to overcome emergent mutations (Milhollen et al. 2012; Toth et al. 2012). In fact, by using a virtual screening approach, a natural product, 6,6″-biapigenin, has been identified and proposed as a second inhibitor of NAE (Leung et al. 2011). In 2012, a cyclometallated rhodium (III) complex was reported as the first metal complex to suppress the NEDDylation pathway via inhibition of NAE, which occupied the same binding pocket as MLN4924 (Zhong et al. 2012).

11.3.2 Experience with Clinical Trials

In parallel with the promising results in preclinical models, MLN4924 has been included in clinical trials for cancer therapy since 2008 (see Table 11.2). Up to now, there are a total of seven Phase I/II clinical trials for MLN4924 in patients with leukaemia, lymphoma, melanoma and several solid tumours. These trials were mainly designed to assess the safety, discomforts and risks of the inhibitor; to establish the maximum tolerated dose (MTD) that can be given to patients; to describe the pharmacokinetics (PK) parameters and pharmacodynamics (PD) effects; to evaluate disease response, and to study MLN4924 in combination with other standard treatments. In general, patients received escalating doses of MLN4924 that was administrated via intravenous infusion on different daily schedules. The pharmacokinetics of MLN4924 were measured in serial blood samples, bone marrow aspirates (BMAs), skin punch biopsies, or fine-needle tumour biopsies collected following drug dosing. The samples were analysed to measure MLN4924-NEDD8 covalent adduct and the expression of CRL substrates, such as CDT1, NRF2, and phospho-IκBα, as the biomarkers to indicate NAE inhibition in peripheral and tumour tissue (Zhao et al. 2014). The PK profiles were similar following dosing on different days, suggesting no apparent accumulation of MLN4924 in plasma with an estimated half-life of 5–15 h. In general, most clinical trials carried out thus far have concluded that MLN4924 is well tolerated in the majority of the dosing schedules studied, with evidence of target inhibition and antitumoural activity which supports continued investigation of MLN4924, both alone and in combination strategies as potential treatment for a variety of human cancers (Nawrocki et al. 2012; Zhao et al. 2014).

11.4 Targeting DUBs

The human genome encodes nearly 100 DUBs in five major classes: 60 Ubiquitin-specific proteases (USPs), 16 Otubain-domain containing proteins (OTUs), 4 Machado-Joseph Domain (Josephin domain)-containing proteins (MJD), 4 ubiquitin C-terminal hydrolases (UCHs) and 8 Jab1/MPN domain-associated metalloisopeptidases (JAMM). USPs, OTUS, MJD and UCHs use an active site cysteine as a nucleophile to attack lysine-glycine isopeptide bonds within ubiquitylated proteins (Nijman et al. 2005), whereas the fifth class of DUBs contains a JAMM zinc metalloproteinase domain (Cope and Deshaies 2003). DUBs are generally expressed as active enzymes, rather than inactive precursors. However, certain DUBs require ubiquitin binding to obtain their active conformation and prevent their uncontrolled proteolytic activity. Structural data revealed that ubiquitin-binding by DUBs is accompanied by active site rearrangements, which are necessary to induce their hydrolytic activity (Edelmann et al. 2009; Komander et al. 2009). DUB activity is also regulated through the binding of scaffold and adaptor proteins (Ventii and

Table 11.2 Clinical trials for MLN4924

Disease type	Clinical status	With combination	MTD	Responses	Ref
Advanced Solid tumors	Phase I	Alone With dexamethasone (Dex)	50 mg/m^2 in Schedule A (days 1-5); 67 mg/m^2 in Schedule B (days 1, 3 and 5 + Dex); 50 mg/m^2 in Schedule C (days 1, 3 and 5)	Stable disease	Clinicaltrials.gov Identifier: NCT00677170 Kauh et al. 2011. J Clin Oncol (ASCO Annual Meeting Abstracts) 29 (suppl; abstr 3013)
Relapsed and/or refractory Hodgkin's Lymphoma, Non-Hodgkin's Lymphoma and Multiple Myeloma	Phase I	Alone	110 mg/m^2 in Schedule A (days 1, 2, 8 and 9) Not reported for Schedule B (days 1,4, 8 and 11) and Schedule C (days 1 and 8) 196 mg/m^2 (twice-weekly schedule of days 1, 4, 8 and 11)	3 partial response and stable disease	Clinicaltrials.gov Identifier: NCT00722488 (Nawrocki et al. 2012) Shah et al. 2010. Blood (ASH Annual Meeting Abstracts) 116: abstract 2801 Harvey et al. 2012 (17th Congress of European Hematology Association) abstract 1060 TrialTroveID-170665

Acute Myeloid Leukemia, Acute Lymphoblastic Leukemia, Myelodysplastic Syndromes	Phase I	Alone With azacitidine	59 mg/m^2 (days 1, 3 and 5)	4 complete responses	Clinicaltrials.gov Identifier: NCT00911066 Swords et al. Blood (ASH Annual Meeting Abstracts) 116: abstract 658 Nawrocki et al. (2012) Zhao et al. (2014)
Metastatic Melanoma	Phase I	Alone	Has not been defined	1 partial response and 9 stable disease	Clinicaltrials.gov Identifier: NCT01011530 Bhatia et al. 2011. J Clin Oncol (ASCO Annual Meeting Abstracts) 29 (suppl; abstr 8529)
Large B-cell Lymphoma	Phase I/II	Alone With standard EPOCH-R chemotherapy	Not reported	Not reported (study was withdrawn before participants were enrolled)	Clinicaltrials.gov Identifier: NCT01415765 Zhao et al. (2014)
Acute Myeloid Leukemia	Phase Ib	Alone With azacitidine	To be determined	Not reported	Clinicaltrials.gov Identifier: NCT01814826 Zhao et al. (2014)
Solid tumors	Phase Ib	With docetaxel With gemcitabine With carboplatin + paclitaxel	To be determined	Not reported	Clinicaltrials.gov Identifier: NCT01862328 Zhao et al. (2014)

Wilkinson 2008), proteolytic cleavages, as well as post-translational modifications (Kessler and Edelmann 2011).

11.4.1 DUBs as Therapeutic Targets

Due to the implication of DUBs in the regulation of crucial signalling pathways such as p53 and NF-κB (Fig. 11.2), it is not surprising that their deregulation is involved in a growing number of diseases, including neurological disorders, viral infections and cancer. Indeed, some members of the DUBs family are known to contribute to neoplastic transformation, such as USP1 (in Fanconi anaemia), USP2 (in prostate cancer), DUB3 (in breast cancer), USP4 (in adenocarcinoma), USP7 (in prostate cancer and non-small-cell lung adenocarcinoma), USP9X (in both leukaemia and myelomas) and BRCC36 (in breast cancer) (Edelmann et al. 2011; Hussain et al. 2009). The genetic alteration of DUBs such as CYLD and USP6, has been associated with skin and bone marrow tumour progression, respectively. Finally, an alteration of expression of A20 (B-cell and T-cell lymphomas), USP10 (carcinomas) and BAP1 (brain, lung and testicular cancers) is observed in some cancers (Hussain et al. 2009; Nicholson et al. 2007; Sippl et al. 2011).

The high degree of substrate specificity and the well defined catalytic pocket make DUBs druggable and amenable to screening with libraries of small molecules (Eletr and Wilkinson 2014). Work carried out over the last few years has led to the identification of inhibitors with selective action against various USP targets, demonstrating the feasibility of selective targeting of DUBs. In general, the initial hits have been obtained by high throughput screening (HTS) of compound libraries, followed by further optimization using structure-activity relationships (Kramer et al. 2012). Companies like Novartis, Progenra and Hybrigenics have patented compounds inhibiting distinct DUBs (see Table 11.3). Some of them will be presented in the following section. However, to the best of our knowledge, only a few of them have, as yet, entered into clinical trials.

11.4.2 DUBs Inhibitors

Recently, a HTS approach led to the identification of two potent and selective reversible inhibitors of the enzymatic activity of the USP1/UAF1 complex, **pimozide** and **GW7646**. USP1, one of the best-characterized DUBs, is a regulator of several important steps in the DNA damage response, mainly in Fanconi anaemia (FA), an hereditary disorder characterized by congenital abnormalities, progressive bone marrow failure, hypersensitivity to DNA cross-linking agents, genomic instability and increased susceptibility to cancer (Li et al. 2002; Reyes-Turcu et al. 2009). USP1 is frequently overexpressed in tumours like cervical and gastric cancer, melanoma and sarcoma. Importantly, USP1 inhibitors act synergistically with the

Table 11.3 Inhibitors of Deubiquitylating enzymes in clinical trials

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Disease type	Ref
Pimozide	Janssen Pharmaceutica	USP1	Diphenylbutylpiperidine	Reversible	Oral	Launched	Tourette syndromeschizophrenia	Chen et al. (2011)
						Phase IV	Movement disorders	Garcia-Santisteban et al (2013) and Zhang and Sidhu (2014) Opler and Feinberg (1991) Neifeld et al. (1983)
						Phase II	Depression, bipolar disorders	
						Phase I	Anxiety	
						Preclinical	Breast cancer, melanoma, non-small-cell lung carcinoma	
6MG	GlaxoSmithKline	PLpro USP14	Thiopurine	Reversible	Oral	Launched	Cancer: Non-Hodgkin's lymphoma, acute lymphatic leukaemia	Chen et al. (2009) Chou et al. (2008) Sippl et al. (2011)
							Other: Psoriatic activity, inflammatory Bowel disease	
						Phase IV	Cancer: Leukemia acute lymphocytic	
							Other: Crohn's disease ulcerative colitis, influenza vaccines, respiratory infections	
						Phase III	Cancer: Non-Hodgkin's lymphoma,	
							Other: HBV, hepatitis vaccine	
						Phase II	Cancer: colorectal	

(continued)

Table 11.3 (continued)

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Disease type	Ref
6TG7	GlaxoSmithKline	PLpro USP14	Thiopurine	Reversible	Oral	Launched	Cancer: acute lymphoblastic leukaemia	Chen et al. (2009) Chou et al. (2008) Sippl et al. (2011)
							Other: inflammatory bowel disease	
						Phase IV	Cancer: Leukemia acute lymphocytic, leukemia acute myelogenous, non-Hodgkin's lymphoma	
							Other: Crohn's disease, ulcerative colitis	
						Phase III	Myelodysplastic syndrome, supportive care	
						Phase II	Cancer: breast, glioblastoma	
P-5091	Progenra	USP7	Tri-substituted thiophene	NS	Unspecified	Preclinical	Multiple Myeloma (MM) and other cancers	Chauhan et al. (2012)
P-022077								
GRL0617	Purdue University	PLpro	5-Amino-2-methyl-N-[(R)-1-(1-naphthyl) ethyl]benzamide	Irreversible	Unspecified	Preclinical	Viral infection	Ratia et al. (2008)
HBX-19, 818	Hybrigenics	USP7	Cyano-pyrazine derivatives	Irreversible	Unspecified	Discovery	Cancer	Reverdy et al. (2012)
HBX-28, 258								

HBX-41, 108	Hybrigenics	USP7	Cyano-indenopyrazine	Reversible	Unspecified	Discovery	Cancer	Reverdy et al. (2012) Zhang and. Sidhu (2014)
IU1	Dan Finley's Laboratory	USP14	1-[1-(4-fluorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-(1-pyrrolidinyl)-ethanone	Reversible	Unspecified	Discovery	Neurodegenerative diseases, Virus infection	Lee et al. (2010) Nag and Finley (2012)
HBX 90,397	Hybrigenics	USP8	Cyano-pyrazine derivatives	NS	Unspecified	Discovery	Cancer	Zhang and Sidhu (2014)
GW7647	GlaxoSmithKline	USP1	Propanoic acid	Reversible	Unspecified	Discovery	Cancer	Chen et al. (2011)

chemotherapy drug cisplatin to inhibit the proliferation of cisplatin-resistant non-small cell lung cancer (NSCLC) cells (Chen et al. 2011; Garcia-Santisteban et al. 2013).

One company, Hybrigenics, has developed a variety of different inhibitors of the USP family (Table 11.3). For example, **HBX41-108** is a cyano-indenopyrazine derived small molecule compound which modulates the catalytic reaction of USP7 (Colland et al. 2009). USP7, also known as herpes virus-associated USP (HAUSP), is critical in cancer progression because of its destabilizing effect on the tumour suppressor p53 (Cheon and Baek 2006; Cummins and Vogelstein 2004). However, USP7 can also modify other substrates such as claspin, FOXO4 and PTEN, suggesting that USP7 exerts both p53-dependent and -independent effects on the control of cell proliferation and apoptosis (Faustrup et al. 2009; Song et al. 2008; van der Horst et al. 2006). HBX41-108 treatments stabilize p53, activate transcription of p53-target genes without inducing genotoxic stress, and inhibit cancer cell growth. Another compound more recently developed by Hybrigenics, **HBX19-818**, is a second-generation of irreversible USP7 inhibitors; it is more specific than HBX41-108, and is capable of regulating USP7 substrates in cancer cells and recapitulating the USP7 knockdown phenotype (Reverdy et al. 2012). Recently, an independent screen performed by Progenra also identified compound **P005091**, and analogues such as **P045204** and **P022077**, as USP7 inhibitors capable of inducing apoptosis in multiple myeloma cells resistant to both conventional and Bortezomib therapies (Chauhan et al. 2012).

Another HTS identified **IU1** as a selective and reversible small-molecule inhibitor of human USP14, which is, together with UCH37 and RPN11, a DUB associated with the proteasome. IU1 binds specifically to the activated form of USP14 and enhances the degradation of several proteins involved in neurodegenerative diseases (like Tau and ataxin-3), suggesting a potential strategy to reduce levels of misfolded and aggregated proteins in cells under proteotoxic stress (Lee et al. 2010; Todi and Paulson 2011).

Inhibitors of DUBs can also be used in antiviral drug development since viruses also encode for DUBs. For example, IU1, mentioned above, is also able to inhibit replication of Dengue virus (Nag and Finley 2012). In the case of Severe Acute Respiratory Syndrome (SARS) caused by SARS-coronavirus, a DUB, called the papain-like protease (Plpro), blocks IRF3-dependent antiviral responses (Edelmann et al. 2011; Mielech et al. 2014). A non-covalent inhibitor of PLpro, **GRL0617**, has been synthesized. This inhibitor modifies the conformation of PLpro, inducing an inhibition of its catalytic activity, and selectively blocks SARS-Coronavirus viral replication without measurable cytotoxic effect (Ratia et al. 2008). Looking for effective therapeutics against severe acute SARS, **6-mercaptopurine** (6MP) and **6-thioguanine** (6TG) were found to be specific inhibitors for PLpro. The potential use of 6MP and 6TG as cellular DUB inhibitors has been further studied. The best docking score and binding energy for 6MP and 6TG is against USP14 (Chen et al. 2009; Chou et al. 2008).

11.4.3 Experience with Clinical Trials

Recently, **Pimozide** and **GW7647** have been identified as potent inhibitors of USP1/UAF1 (Chen et al. 2011). Pimozide is an antipsychotic drug also known for its neuroleptic property in the treatment of patients with schizophrenia (Opler and Feinberg 1991), Tourette syndrome and resistant tics. In addition, pimozide inhibits the proliferation of human melanoma and breast cancer cells (Neifeld et al. 1983; Strobl 1990) and is able to reverse the chemo resistance of non-small cell lung cancer cells to the DNA cross-linker, cisplatin, further supporting USP1/UAF1 as a potential target for novel anticancer therapy (Chen et al. 2011) (Table 11.3).

6MP6 and 6TG7 are antimetabolite antineoplastic agents with immunosuppressive properties, interfering with nucleic acid synthesis by inhibiting purine metabolism. These antimetabolites, also described as PLpro and potential USP14 inhibitors, are usually used in combination with other drugs, in the treatment of or in remission-maintenance programs for leukaemia. Several clinical trials have been established or are currently in progress using 6MP6 (166 trials listed in www.clinicaltrial.gov) and 6TG7 (66 trials listed in www.clinicaltrial.gov), in combination with other chemotherapeutic drugs, for the treatment of leukaemia, brain tumour, breast and ovarian cancer, as well as immune diseases.

11.5 Targeting Ubiquitin Ligases

Several specific E3 ubiquitin ligases have been reported as deregulated or overexpressed in various diseases and cancers. Here, we will describe inhibitors of specific E3 ligases already in preclinical development or in phase I clinical trials (see Table 11.4). However, due to the importance of the E3 ligase Mdm2 in the regulation of the tumour suppressor p53, many small molecule inhibitors of this protein-protein interaction were initially developed.

11.5.1 Inhibitors of p53/Mdm2

The tumour protein, p53, is involved in a broad range of critical pathways such as cell cycle, apoptosis, senescence, DNA repair, metabolism, development and innate immunity (Vousden and Prives 2009). p53 monitors the integrity of the genome and is tightly regulated by different intracellular pathways, its main regulator being the ubiquitin E3 Hdm2 ligase (Mdm2 in mouse) that is involved in an auto-regulatory loop controlling p53 stability and activity. The transcription factor p53 has been found mutated and/or deregulated in about half of all known cancers (Soussi and Beroud 2001). In other cases, p53 remains functional but abnormally retained by overexpressed or mutated Mdm2, or its homologue MdmX/Mdm4.

Table 11.4 Inhibitors Ubiquitin E3 ligases in clinical trials

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Cancer type	Ref
MK-8242	Merck & Co	p53-mdm2 interaction	Nutlins	Reversible	Oral	Phase I	Cancer: Leukaemia acute myelogenous, prostate, soft tissue sarcoma, unspecified solid	www.clinicaltrials.gov
SAR-405838	Ascenta Therapeutics	p53-Hdm2 interaction	Spirooxindole	Reversible	Oral	Phase I	Cancer: Non-Hodgkin's lymphoma, Hodgkin's lymphoma, soft tissue sarcoma, unspecified solid	Shen et al. (2013)
RO-5503781	Hoffmann-La Roche	p53-Hdm2 interaction	Nutlins	Reversible	Oral	Phase I	Cancer: Leukaemia acute myelogenous, prostate, unspecified solid	Wang et al. (2011)
DS-3032	Daiichi Sankyo	p53-mdm2 interaction	Imidazothiazole	Unspecified	Oral	Phase I	Cancer: Non-Hodgkin's lymphoma, Hodgkin's lymphoma, melanoma, unspecified solid	www.clinicaltrials.gov

CGM-097	Novartis	p53-Hdm2 interaction	Unspecified	Unspecified	Oral	Phase I	Cancer: advanced solid tumours. Patients that do not respond to standard therapies	www.clinicaltrials.gov
TL-32711 (birinapant)	TetraLogic Pharmaceuticals	IAP	Propanamide	Unspecified	IV	Phase III	Cancer: colorectal	www.clinicaltrials.gov
							Myelodysplastic syndrome	
						Phase II	Cancer: Non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukaemia acute myelogenous, non small cell lung, small cell lung, melanoma, ovarian, pancreas, unspecified solid	
						Phase I	Cancer: breast	

(continued)

Table 11.4 (continued)

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Cancer type	Ref
AT-406	Ascenta Therapeutics	XIAP, IAP1 and IAP2	Carboxamide	Unspecified	Oral	Phase II	Cancer: head and neck	www.clinicaltrials.gov
						Phase I	Cancer: Non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukaemia acute myelogenous, non-small cell lung, breast, ovarian, unspecified solid	
LCL-161	Novartis	IAP	Unspecified	Unspecified	Oral	Phase II	Cancer: multiple myeloma, breast	www.clinicaltrials.gov
						Phase I	Cancer: pancreas, ovarian, colorectal, unspecified solid	
HGS-1029	Pharmascience	IAP2	Hydrochloride salt	Unspecified	IV	Phase I	Cancer: Non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukaemia acute lymphocytic, leukaemia chronic lymphocytic, non small cell lung, breast, colonorectal, head and neck, pancreas, prostate, unspecified solid	www.clinicaltrials.gov

PO13222	Progenra	MuRF1	Unspecified	Reversible	Unspecified	Preclinical	Cancer Others: Prevention of muscle atrophy, AIDS, congestive heart disease, severe burns	Eddins et al. (2011)
E6AP Inhibitor	Cancer Therapeutics CRC	E6AP	Unspecified	Unspecified	Unspecified	Preclinical	Cancer: HPV-related	Dymalla et al. (2009)
AM-8735	Amgen	p53-Hdm2 interaction	Morpholinone	Unspecified	Oral	Preclinical	Cancer unspecified	Gonzalez et al. (2014)
ATSP-7041	Aileron Therapeutics Hoffmann-La Roche	Dual p53-Mdm2 p53-Mdmx	α–helical peptides	Unspecified	Unspecified	Preclinical (Phase I planned)	Cancer: breast, colon Liposarcoma, melanoma, solid tumors Others: AML, CML	Chang et al. (2013)
PXN-527 PXN-523	Priaxon Nexus Pharma	p53-Mdm2 interaction	Nutlins	Reversible	Oral	Preclinical	Cancer unspecified	Cheok et al. (2011)

(continued)

Table 11.4 (continued)

Drug name	Company	Molecular target	Family structure	Binding type	Adms route	Clinical status	Cancer type	Ref
SIAH inhibitors	Cancer Therapeutics	SIAH protein interaction	Unspecified	Unspecified	Unspecified	Preclinical	Cancer: pancreas, breast, ovarian, prostate	Stebbins et al. (2013)
APN 401	Apeiron Biologics	Cbl-b	Nucleic acid: RNA interference	Unspecified	Unspecified	Preclinical	Cancer unspecified	Bachmaier et al. (2000)
Synoviolin inhibitor	Progenra	Synoviolin	Unspecified	Unspecified	Unspecified	Preclinical	Rheumatoid, Arthritis(RA)	Yagishita et al (2012)
T-3256336	Takeda	IAPs	Pyrazine scaffolds	Unspecified	Oral	Preclinical	Cancer unspecified	www.clinicaltrials.gov
IAP antagonist	Takeda	IAPs	Pyrazine scaffolds	Unspecified	Unspecified	Preclinical	Cancer unspecified	www.clinicaltrials.gov
IAP antagonist	Hoffmann-La Roche	IAPs	Benzoxazepinon	Unspecified	Unspecified	Preclinical	Cancer unspecified	www.clinicaltrials.gov
AZ7732	AstraZeneca	IAPs	Beta-alanine-derived	Unspecified	Unspecified	Preclinical	Cancer unspecified	www.clinicaltrials.gov
AT-IAP	Astex Pharmaceuticals	IAPs	Unspecified	Unspecified	Unspecified	Preclinical	Cancer unspecified	www.clinicaltrials.gov

In 1996, the high-resolution crystal structure of Mdm2 with p53 was published (Kussie et al. 1996) and more recently, the MdmX/p53 crystal structure was resolved (Popowicz et al. 2008). From these studies, non-peptide small molecules inhibitors have been designed to specifically target the hydrophobic pocket including three trivial amino acids (Phe19, Trp23 and Leu26) common to both Mdm2 and MdmX. Hoffmann-La Roche has been the first company to develop one of the most potent Mdm2/p53 inhibitors, namely Nutlins. There are three generations of **Nutlins** (Nutlin-2; Nutlin-3a; RG-7112), with each generation aiming to improve the stability, affinity and bio-availability of the inhibitor. After optimization of Nutlin-3a, RG-7112 was the first Mdm2 inhibitor used in clinical trials to treat multiple human cancers (Tovar et al. 2013). Other companies like Amgen developed a series of optimized compounds from the **piperidinone** class of compounds, obtaining **AM-8553** that efficiently inhibits tumour regression (Rew et al. 2012). Since 2013, Amgen also developed the Mdm2 inhibitor, **AMG-8735**, containing a morpholinone core with a significant increase in both potency and metabolic stability compared to the piperidinone series. AMG-8735 emerges as an inhibitor with remarkable biochemical potency and pharmacokinetic properties (Gonzalez et al. 2014) (Table 11.4).

ATSP-7041, **developed by Aileron Therapeutics**, is the first highly potent and selective stapled α-helical peptide that functions as a dual inhibitor of Mdm2 and MdmX. ATSP-7041 binds to Mdm2 and MdmX with nanomolar affinities and restores p53 activity (Chang et al. 2013). ATSP-7041 has a potential use for the treatment of solid tumours and cancers like AML, CML, breast cancer, liposarcoma, melanoma or colon cancer. Stapled α-helical peptides emerged as a promising new modality for a wide range of therapeutic targets. Based on Nutlin-3a (Roche), MI-219 (Ascenta) and TDP 222669 (Johnson&Johnson) structures, and using an NMR spectroscopy approach as an *in silico* compound-selection process, Priaxon AG developed two new classes of MDM2-inhibitors, **PXN-527** and **PXN-523**. These inhibitors have been tested in cellular cultures for inhibition of proliferation assays and are in preclinical trials (Cheok et al. 2011).

11.5.2 Inhibitors of Other E3 Ubiquitin Ligases

In addition to Mdm2/MdmX inhibitors, other inhibitors of ubiquitin E3 ligases are also in clinical trials. Progenra developed **PO13222**, a compound capable of specifically inhibiting the E3 ubiquitin ligase, MuRF1, a ligase associated with muscle wasting diseases. PO13222 specifically inhibits auto-ubiquitylation activity, without affecting ubiquitin E1 and E2 enzymes, resulting in the stabilization of muscle protein. This product is in preclinical development since 2012, the aim of which is to provide a novel therapeutic strategy to protect muscles from sarcopenia (aging) and atrophy associated with common diseases (such as cancer, AIDS and congestive heart disease) and severe burns (Eddins et al. 2011) (Table 11.4).

Progenra has also developed **Synoviolin/hrd1 inhibitors** for the treatment of rheumatoid arthritis (RA). Synoviolin is a RING E3 ubiquitin ligase implicated in

the endoplasmic reticulum-associated degradation (ERAD) pathway. Synoviolin is highly expressed in rheumatoid synovial cells and possibly involved in the pathogenesis of RA. Using HTS, two classes of small molecules have been identified that efficiently inhibit synoviolin activity by inhibition of autoubiquitination enzymatic activity, thus blocking proliferation of synovial cells. This inhibitor has been in preclinical development since 2011 (Yagishita et al. 2012).

High-risk oncogenic human papillomavirus (HPV) types (including HPV 16 and HPV 18) are associated with 99.7 % of all cervical cancers. The E6 and E7 oncoproteins are critical factors that maintain the malignant phenotype of HPV tumour cells. By using yeast two-hybrid screening, Cancer Therapeutics CRC developed linear short peptides that specifically bind with high affinity to the HPV16 E6 oncoprotein, affecting its interaction with the HECT-E3 ubiquitin ligase E6-AP (also known as UBE3A) that degrades p53. The restoration of intracellular p53 by using these short peptides is a new strategy for the treatment of HPV cancers. This E6AP inhibitor is in preclinical stage studies since 2012 (Dymalla et al. 2009).

The E3 ubiquitin ligase Cbl-b is a key regulator of activation thresholds in mature lymphocytes, and of immunological tolerance and autoimmunity. Apeiron Biologics developed the specific Cbl-b ligase inhibitor, **APN-401**, that stimulates immune cells, providing a novel way to treat cancer more effectively. This compound is under preclinical development since 2012 (www.clinicaltrials.gov).

The SIAH (Seven in Absentia Homologue) family of RING-E3 ubiquitin ligases regulates cellular events that are critical for cancer development and progression. Cancer Therapeutics developed a covalent peptides-based inhibitor (Cys-trapping moiety) to disrupt SIAH interactions with adaptor proteins, and thereby block cancer progression. These **small molecule SIAH inhibitors** are currently being tested in a preclinical phase for patients with breast, ovarian, prostate and pancreas cancers (Stebbins et al. 2013).

11.5.3 Experience with Clinical Trials

The experience with ubiquitin E3 inhibitors in clinical trials is limited compared to proteasome inhibitors. Many of them are in phase I of clinical development and primary outcomes such as maximum tolerated dose, dose-limiting toxicities and safety are currently being evaluated. Subsequently, efficacy, pharmacokinetics and pharmacodynamics will be measured as secondary outcomes.

After the generation of the first inhibitor for Mdm2, RG-7112, Hoffmann-la Roche developed another Mdm2 inhibitor, **RO-5503781**, whose structure has not been published. This orally available inhibitor has been in phase I clinical trials since 2011 (NCT01462175), and is proposed to cancer patients with solid tumours and acute myelogenous advanced malignancies, except in the case of leukaemia. However, with respect to the latter, RO-5503781 has been included in another trial in combination with cytarabine, a chemotherapeutic agent used for treatment of leukaemia and non-Hodgkin lymphoma. A third trial is under consideration where RO5503781 will be used in association with cytarabine and anthracycline.

SAR-405838 is a small analogue molecule of MI-888 (small molecule inhibitors of Mdm2), developed by Ascenta Therapeutics and Sanofi, that is currently in phase I of clinical trials since 2012, either alone (Clinical trial: NCT01985191) or in combination with Pimasertib (Clinical trial: NCT01636479). This oral drug may be used for patients with malignant neoplasm (Shen et al. 2013).

CGM097 is an oral HDM2/p53 inhibitor developed by Novartis for the treatment of cancer with advanced solid tumours, or for patients that have regressed despite standard therapy, or for whom no standard therapy exists. Cells have to exhibit wild type p53 to respond to this drug. CGM097 has been in phase I clinical trials since 2012 (NCT01760525).

Merck developed the **MK-8242** inhibitor that is included in phase I clinical trials for patients with advanced solid tumours when there is no other treatment available, or for recurrent acute myelogenous leukaemia and liposarcomas. Like RO5503781, MK-8242 is also being evaluated in phase I clinical trials in combination with cytarabine.

DS3032b is an oral Mdm2 inhibitor that has been developed by Daïchi Sankyo to treat patients with advanced solid tumours or lymphomas that exhibit mutated Mdm2 and a WT p53. The description of this molecule has not yet been published but it has already been included in phase I clinical trials (NCT01877382).

11.6 Conclusions and Perspectives

Even if the first pieces of the puzzle of this ATP-dependent pathway were discovered more than 30 years ago, every year new components and regulatory mechanisms controlling this system are being described. Despite this brief didactic view of the UPS classifying enzymes, cofactors and substrates, the system is a lot more complex than presented here, and there is still a distinct lack of knowledge with respect to its overall organization. For instance, it is well known that under certain circumstances several modifying/de-modifying enzymes can act on distinct members of the ubiquitin family (Leidecker et al. 2012), that proteasome subunits can be substrates of ubiquitin and ubiquitin-like molecules (Cui et al. 2014), or that the proteasome can also drive proteolysis in the absence of protein modification (Erales and Coffino 2014). Although most of the functional outcomes of recent findings are not completely understood, it becomes clear that simply targeting a single enzyme-substrate pair will be difficult. However, this should not stop the use of these inhibitors to treat distinct pathologies, as has recently occurred with proteasome inhibitors that block multiple processes, since the discovery of drugs acting at different levels will also increase the chances of clinical success. Furthermore, the experience using combinations of different inhibitors is quite positive in cases where patients do not respond or are resistant to the current treatments. Although these combinatorial drug therapies might appear to be in contradiction with the initial aim of reducing undesirable side effects, it is possible that eventually they could be more effective. A compromise between efficacy and cytotoxicity should be better defined by

pharmacodynamics and pharmacokinetic studies. Finally, although we described the use of UPS inhibitors to treat some cancers, many of them have also been used to treat quite diverse diseases such as cardiac, immune and neurodegenerative disorders. The use of chemical inhibitors will contribute to a better understanding of the multiple cellular pathways regulated by the UPS. More importantly, these inhibitors will open up new perspectives for the treatment of various diseases, as has been the case for Bortezomib/Velcade (reviewed by Xolalpa et al. 2013).

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Chapter 12
Endoplasmic Reticulum Stress in Multiple Myeloma: From Molecular Mechanisms to Therapeutic Opportunities

Bei Liu and Zihai Li

Abstract The endoplasmic reticulum (ER) is a membrane-bounded intracellular organelle with an essential role in protein synthesis, folding and transport. Accumulation of misfolded proteins in the ER leads to ER stress, which triggers the activation of three well-known pathways including activating inositol requiring kinase 1 (IRE1), the transcription factor activating transcription factor 6 (ATF6), and double stranded RNA-activated protein kinase-like ER kinase (PERK) to induce the expression of several major ER heat shock proteins (HSPs) including gp96, grp78 and calreticulin to enhance protein folding machinery. These signaling pathways are termed unfolded protein response (UPR), which are critical for cell fates. Multiple myeloma (MM) is an incurable plasma cell neoplasm whose pathogenesis is closely linked to dysregulated UPR in ER due to the heightened production of immunoglobulin and the metabolic demands of malignant uncontrolled proliferation. Therefore, inhibition of the ER stress response is likely to injure the MM cells, as is any further demand on an already over-worked system. In this chapter, we discuss the roles of ER stress sensors in plasma cell differentiation and MM pathogenesis. We also summarize the strategies of targeting UPR pathways and HSPs that have been proposed and tested for potential therapeutic benefit against multiple myeloma.

Keywords Endoplasmic reticulum stress • Unfolded protein response • Activating inositol requiring kinase 1 • Transcription factor activating transcription factor 6 • Double stranded RNA-activated protein kinase-like ER kinase • Multiple myeloma • Apoptosis • Chaperone • Heat shock protein • Bortezomib • ER chaperone gp96 • grp78 • Calreticulin • HSP70

B. Liu (✉) • Z. Li (✉)
Department of Microbiology and Immunology, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425, USA
e-mail: liube@musc.edu; zihai@musc.edu

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12.1 Introduction

Multiple myeloma (MM) is a malignant plasma cell disorder and is the second most common hematological malignancy in the United States. Approximately 24,050 new cases of MM will be diagnosed in 2014 in the US with 11,090 deaths. The tumorigenic plasma cells highly secrete monoclonal immunoglobulin and induce a wide range of pathologies including lytic bone disease, hypercalcemia, immune dysfunction, anemia and kidney failure (Anderson and Carrasco 2011). MM almost always derives from a benign condition called monoclonal gammopathy of undetermined significance (MGUS). Patients could present as asymptomatic smoldering MM phase, then progresses to advanced symptomatic phases of MM, which include an active, relapsing and refractory periods (Boyd et al. 2012). MM typically presents as an incurable disease, almost inevitably recurring after therapy (Munshi and Anderson 2013). However, the introduction of the proteasome inhibitor bortezomib to the treatment regimens represented a breakthrough for myeloma patients by increasing survival time significantly (Moreau 2012). The sensitivity of myeloma cells to bortezomib may be due in part to the specialized metabolism of plasma cells, which are adapted to generate large volumes of secreted immunoglobulins and operate with an elevated baseline demand on the endoplasmic reticulum (ER). This may be a liability for myeloma cells, which are additionally burdened with the protein production necessary for malignant proliferation. The resulting vulnerability to further perturbation in protein metabolism may offer a partial explanation for the success of bortezomib (Landowski et al. 2005; Meister et al. 2007; Obeng et al. 2006). Efforts to understand and target the integrated ER stress response in MM will be summarized here, with a focus on the three ER stress sensors that coordinate this response: inositol requiring kinase 1 (IRE1) (Sidrauski and Walter 1997; Yoshida et al. 2001), the transcription factor activating transcription factor 6 (ATF6) (Yoshida et al. 2000), and double stranded RNA-activated protein kinase-like ER kinase (PERK) (Harding et al. 2000). Each of these sensors is located at the apex of a pathway, and each is capable of inducing the expression of several major ER HSPs and enhancing protein folding machinery (Malhotra and Kaufman 2007) (Fig. 12.1).

All three ER stress response sensors are embedded in the ER membrane where they are normally bound by the ER chaperone grp78 (alias BiP) (Kimata et al. 2004; Ma et al. 2002; Sommer and Jarosch 2002). This binding inhibits the activity of each sensor. Grp78 releases the sensors in response to mounting ER stress as its chaperone functions are required (Lee 2005). However, this is not a uniform method of control over the three combined sensors; different cellular conditions result in differing patterns of sensor activation. For example, during B cell differentiation only two sensors, IRE1 and ATF6 are activated while the PERK is not (Ma et al. 2010). Using a B cell line capable of induction of all three ER stress sensors and capable of differentiation into plasma cells, Ma et al. demonstrated that IRE1 is activated quickly upon exposure to differentiation-inducing LPS treatment, with ATF6 activation following secondarily. In contrast, PERK activation could not be

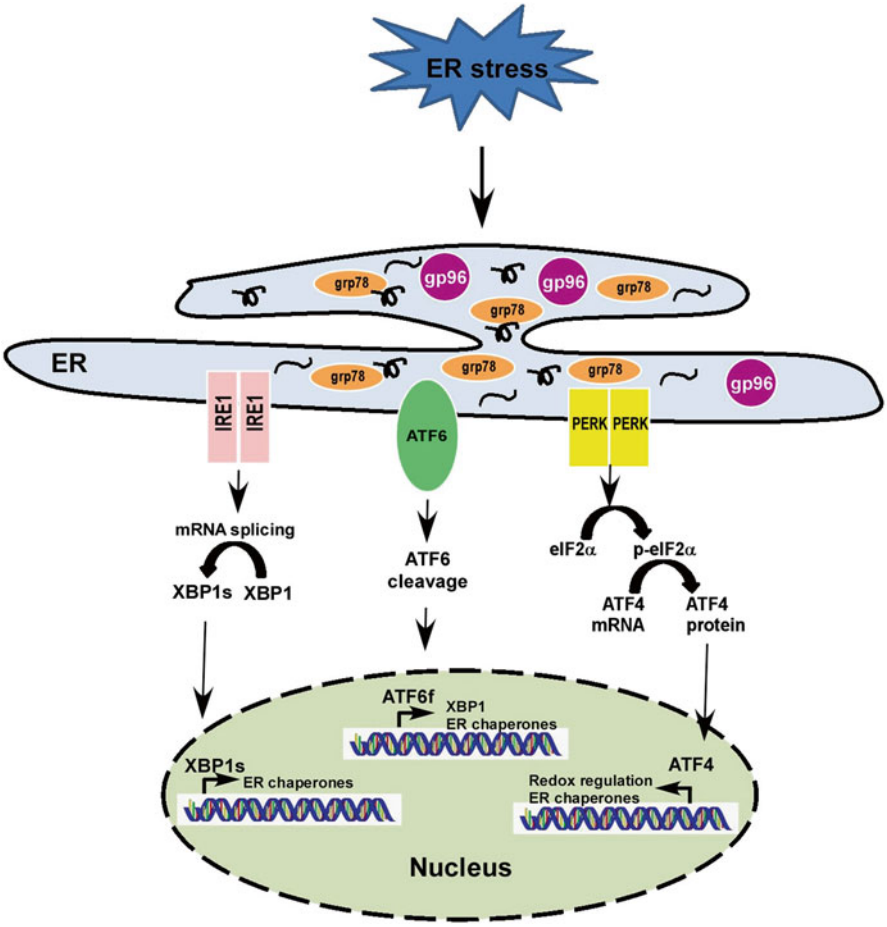

Fig. 12.1 ER stress and unfolded protein response signaling pathway. Upon accumulation of unfolded or misfolded proteins in the ER, three major ER stress sensors, IRE1, ATF6, and PERK are activated following their dissociation from the ER chaperone grp78. Each ER stress sensor is capable of inducing the expression of several major ER chaperones and enhancing protein folding machinery

elicited from these cells upon differentiation, even when treated with the ER stressor thapsigargin, although this treatment could stimulate PERK activity before differentiation (Ma et al. 2010).

Crosstalk between the sensor systems provides additional control over the cellular response. For example, one effect of IRE1 activation is the transcription of a PERK inhibitor named p58ipk (Iwakoshi et al. 2003; Ma et al. 2010). In addition, ATF6 and PERK appear to converge on signaling through the transcription factor CHOP (Okada et al. 2002). Thus, both re-enforcement and antagonism exist

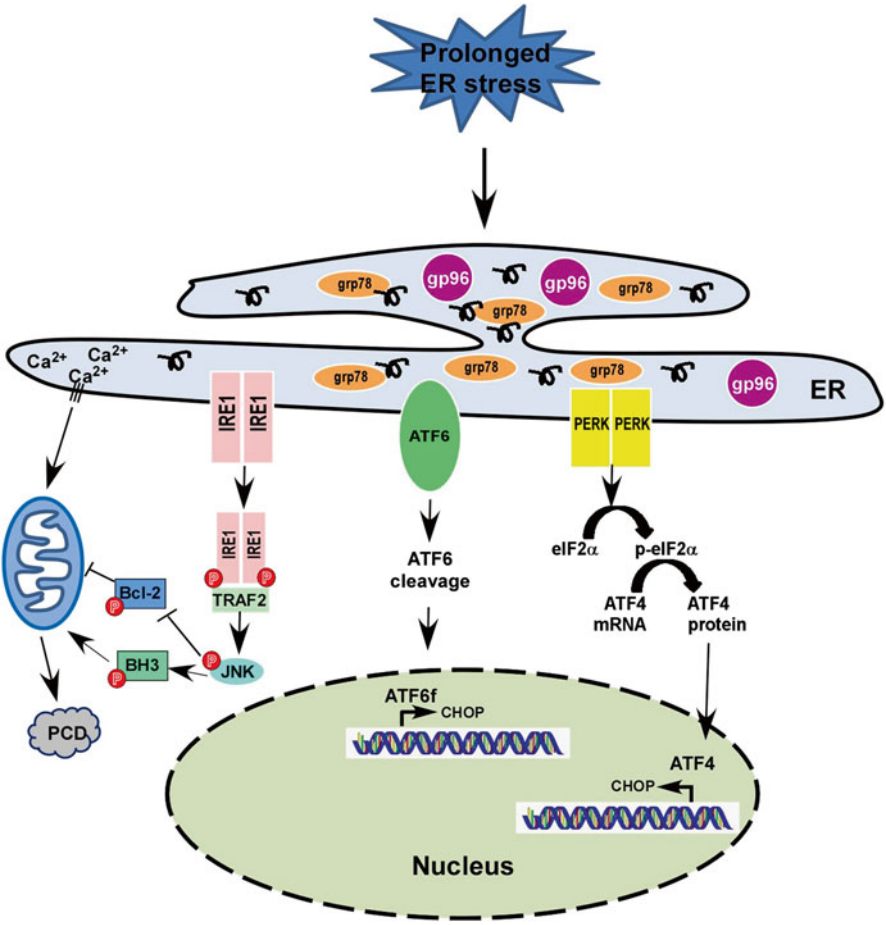

Fig. 12.2 Prolonged ER stress and programmed cell death signaling pathway. Upon persistent ER stress, tipping the system towards programmed cell death (PCD). ATF4 and ATF6 coordinate to induce transcription of CHOP, a pro-apoptosis transcription factor, the ER stress response moves from adaptive to destructive. IRE1 is phosphorylated and binds to TRAF2, then further active JNK-BH3 pathway and induces PCD

between the sensors, allowing a highly tunable response based on cellular needs. Upon the mild ER stress, ER stress sensors activate the adaptive UPR. However, if UPR stress persists, some of the same UPR sensors activate an ER stress-induced programmed cell death (ER-PCD) (Fig. 12.2).

Due to the baseline ER stress present in untransformed plasma cells, myeloma is a particularly complex disease in which to examine ER stress. In this chapter, we discuss the roles of ER stress sensors in normal plasma cell differentiation as well as MM pathogenesis. We also summarize the strategies of targeting UPR pathways and HSPs for potential therapeutic benefit against multiple myeloma.

12.2 The Role of ER Stress in Multiple Myeloma: Molecular Mechanisms

Multiple myeloma (MM) is an incurable plasma cell neoplasm. As multiple myeloma cells actively synthesize and secrete immunoglobulin, they are prone to ER stress and require UPR for survival. In this section, we provide brief overviews of the UPR signaling in myeloma disease.

12.2.1 IRE1 and Multiple Myeloma

12.2.1.1 Overview

IRE1 is a bifunctional transmembrane kinase and endoribonuclease. It was first identified in yeast, called Ire1p, which is implicated in unfolded protein response (UPR) (Cox et al. 1993). Upon activation of the UPR, Ire1p oligomerizes, phosphorylates and initiates splicing of HAC1 (Shamu and Walter 1996; Sidrauski and Walter 1997). IRE1 is conserved in all eukaryotic cells. In mammalian cells, there are two forms of IRE1, IRE1α and IRE1β. Most cells and tissues express IRE1α, while only intestinal epithelial cells express IRE1β. IRE1α and IRE1β have similar cleavage specificities (Patil and Walter 2001; Tirasophon et al. 1998; Wang et al. 1998). Previous studies have demonstrated that *X box binding protein 1 (XBP1)* mRNA is a substrate for the endoribonuclease activity of IRE1. Upon activation of the UPR, the IRE1 RNase activity initiates and removes a 26 nucleotide intron from *XBP1* mRNA (Calfon et al. 2002; Lee et al. 2002; Yoshida et al. 2001). This splicing form of XBP1, denoted XBP1s, is a transcriptional activator that plays an important role in activation of a variety of UPR target genes, including ERdj4, $p58^{IPK}$, DnaJ/Hsp40-like genes, EDEM, HEDJ, protein disulfide isomerase-P5 (PDI-P5), and ribosome-associated membrane protein 4 (RAMP4) (Lee et al. 2003).

12.2.1.2 IRE1/XBP1 Pathway Is Essential for Plasma Cell Differentiation

Both IRE1 and XBP1 are critical for plasma cell differentiation. Genetic deletion of XBP1 causes lack of plasma cells, with concomitantly decreased baseline and antigen specific serum level of immunoglobulin (Iwakoshi et al. 2003; Reimold et al. 2001; Shaffer et al. 2004). In addition, IRE1α is required to splice XBP1 for terminal differentiation of mature B cells into antibody-secreting plasma cells as demonstrated by using an IRE1α-deficient chimeric mouse model (Zhang et al. 2005). Furthermore, in IRE1α conditional knockout mice, the serum levels of IgM and IgG1 are reduced by half compared with the control mice. However, the IgM^+, IgD^+ and $B220^+$ populations are similar between IRE1α conditional knockout mice and control mice. This result suggests that IRE1α is required for efficient plasma cell production of antibodies, and is critical for final B cell differentiation into a plasma

cell (Iwawaki et al. 2010). Very interestingly, the role of XBP1 in plasma cell differentiation does not appear to be due to increased Ig synthesis. It is required for optimal B cell receptor signaling as well as migration of plasmablast to the proper niche in the bone marrow (Hu et al. 2009). These studies suggest that the IRE1/XBP1 pathway is required for differentiation and survival of plasma cells.

12.2.1.3 IRE1/XBP1 Pathway in Multiple Myeloma Pathogenesis

In addition to the critical role of UPR in plasma cell differentiation, XBP1s and the downstream ER chaperones are consistently upregulated in myeloma patients (Bagratuni et al. 2010). High levels of spliced *XBP1* mRNA were consistently detected in all 253 newly diagnosed MM patients, and high ratios of spliced versus unspliced *XBP1* mRNA (>1.33) directly correlated with lower overall survival. Recent study showed that low *XBP-1* levels predicted poor response to bortezomib, both in vitro and in MM patients. Moreover, selected bortezomib resistance MM cell lines down-regulate *XBP-1* and immunoglobulin secretion (Ling et al. 2012). These findings suggest that XBP1 might play an important role in MM pathogenesis. Indeed, transgenic expression of XBP1s in mice also leads to plasma cell dyscrasia with evidence of increased monoclonal antibodies ('M-spike'), lytic bone lesions, plasmacytosis and kidney damage (Carrasco et al. 2007). This study shows that XBP1 overexpression alone can drive transformation of plasma cells and promote multiple myeloma pathogenesis, underscoring the importance of dysregulated UPR in malignancy.

12.2.2 ATF6 and Multiple Myeloma

12.2.2.1 Overview

Among the three ER stress sensors, ATF6 does not dimerize to potentiate enzymatic activity. Instead, under ER stress conditions, ATF6 translocates to the Golgi apparatus and it is processed by site 1 protease (S1P) and site 2 protease (S2P) to release an active form of ATF6 (ATF6f). ATF6f translocates to the nucleus and activates target genes (Chen et al. 2002). In this capacity, ATF6 works in partnership with IRE1, as one of the target genes of ATF6 is XBP1, the key substrate of IRE1 (Yoshida et al. 2001). In addition to fueling the IRE1 arm of the ER stress response, ATF6 also functions as a transcription factor for ER chaperone proteins, thereby easing ER burden (Arai et al. 2006). These contributions to the ER stress response complement IRE1 activation and are generally adaptive, allowing such upregulation of protein production as is seen in plasma cell development. However, prolonged ATF6 activation can also result in transcription of CHOP, another transcription factor which enacts a largely apoptotic program of gene expression (Matsumoto et al. 1996). This effect of ATF6 activity occurs in conjunction with PERK activation, in contrast to the protective program that ATF6 and IRE1 jointly support.

One group has made an attempt in HeLa cells to describe the genetic modulation downstream of ATF6 activation and to distinguish it from the genetic signature of PERK activation (Okada et al. 2002). The group examined this question by comparing the cellular pool of mRNA in HeLa cells treated with the general ER-stress inducer tunicamycin with that of cells stably expressing the nuclear form of ATF6. From this experiment, the ATF6 contribution to the integrated ER stress response was extracted for HeLa cells. The primary targets identified were the expected ER chaperones grp78, gp96, and calreticulin (Okada et al. 2002). In addition, proteins which directly modify disulphide bonds to assure proper folding of nascent proteins were identified, such as ERp62 and ERp71 (Okada et al. 2002). Unfortunately, the authors concluded that this cell system was not conducive to the study of XBP1 transcription, which is critical for understanding myeloma development and progression. However, the research revealed that ATF6 and PERK both converge on CHOP transcription, confirming this as a locus of crosstalk between the two sensors (Okada et al. 2002).

CHOP (C/EBP homologous protein, alias GADD153) is a pro-apoptotic transcription factor routinely used as a read-out for activation of the ER stress response (Kawabata et al. 2012; Mimura et al. 2012; Schonthal 2013). The Mori group has proposed that CHOP transcription is most efficiently activated upon binding by both the nuclear form of ATF6 and ATF4, the transcription factor effector of PERK activation (Okada et al. 2002). The convergence of ER stress signals results in CHOP binding to its target genes, with inhibitory effects on some targets and transcriptional effects on others. CHOP activity results in the downregulation of the anti-apoptotic Bcl2 as well as the upregulation of the ER-resident oxidase ERO1-alpha (Marciniak et al. 2004). CHOP is also its own target, suggesting that its activation constitutes a commitment to programmed cell death (Marciniak et al. 2004).

12.2.2.2 ATF6 in MM

Surprisingly little has been reported about the role of ATF6 in MM, especially considering the important role it plays in the generation of the IRE1 substrate XBP1 (Lee et al. 2002). Indeed, the transcriptome of ATF6 should itself be a discrete target of research in the myeloma field.

Recent study showed that specific knockdown of ATF6 in MM cells resulted in significant cell death, and as is also the case for the other ER stress sensors (IRE1 and PERK) (Michallet et al. 2011). In addition, increased baseline signaling through the PERK sensor was enhanced upon knockdown of ATF6. Thus, the three sensors appear to all be required for baseline survival for MM cells, although crosstalk may allow for some limited compensation between the sensor systems.

Certainly, the crosstalk between ATF6 and the other two ER stress sensors suggests that ATF6 plays the role of a "swing vote." When activated in conjunction with IRE1, growth and adaptation to protein production is reinforced. When linked to PERK, ATF6 activity can support an ER programmed cell death response. This duality indicates a potentially powerful target, identifying ATF6 as an understudied aspect of myeloma.

12.2.3 PERK and Multiple Myeloma

12.2.3.1 Overview

The Pancreatic eIF2-alpha kinase (PERK, alias EIF2αK3) is the third known sensor of ER stress and like the other two, it is embedded in the ER membrane. As the only such sensor left inactivated in the normal development of plasma cells, it has been of particular interest in the study of myeloma (Ma et al. 2010). We will therefore provide a summary of its canonical function and then review studies testing the role of PERK in baseline myeloma biology and in response to drug treatment.

Like the other two ER stress sensors, the activation of PERK requires its release by ER chaperone grp78. In addition, the other ER chaperone gp96 (alias grp94) has been shown to bind PERK at baseline and release it during ER stress conditions (Ma et al. 2002). Upon release, PERK is free to homodimerize and activate as a kinase. Active PERK has three interacting mechanisms, allowing gradations of cellular effects ranging from protective to destructive. These effects are mediated by eIF2-alpha, ATF4, and CHOP. First, the direct phosphorylation target of PERK is eIF2-alpha, a protein needed for ribosomal translation of mRNA (Wek and Cavener 2007). The phosphorylation of eIF2-alpha inhibits its activity, resulting in global repression of protein production. This strategy of translation repression reduces the load of nascent proteins being delivered to the ER for processing and is an effective short term answer to the problem of ER stress. However, the side effects of halting protein production are myriad, and the phosphorylation of eIF2-alpha does allow exceptions. For instance, mRNA with IRES sequences can still be translated under these conditions (Gerlitz et al. 2002). In addition, the transcription factor ATF4 is translated and subsequently translocated to the nucleus. The mechanism allowing such translation during eIF2α phosphorylation has been of significant interest and research has identified a double upstream open reading frame structure in the *ATF4* mRNA which is preferentially translated when ribosomal processing is slowed (Kilberg et al. 2009; Lu et al. 2004). ATF4 then binds to genetic sequences with CCAAT motifs, many of which can be translated under the phosphorylated eIF2a condition which is downstream of PERK activation, likely due to upstream ORFs that function like the ones present in *ATF4* mRNA (Kilberg et al. 2009; Lu et al. 2004). This activation of the ATF4 transcriptome is the second major arm of PERK response to ER stress.

ATF4 facilitates the transcription mRNAs coding for proteins with functions specific to ER stress conditions. For instance, redox-management genes are turned on, as well as additional chaperones for the ER (Harding et al. 2003; Liu et al. 2008; Ye and Koumenis 2009). Again, this strategy is adaptive for the cell and may allow the cell to cope with short term ER stress. However, the third arm of PERK signaling involves activation of CHOP, already described as a target of ATF6. The CHOP promoter includes binding sites for both ATF4 and ATF6, which appear to synergize (Okada et al. 2002). In addition, the *CHOP* mRNA includes an upstream inhibitory ORF that is preferentially translated during ER stress (Jousse et al. 2001; Lee et al. 2011). Expression of this protein is very tightly regulated and eventual convergence

on CHOP activation signals a likely shift into macroautophagy and/or apoptosis (Emdad et al. 2011; Gomez-Santos et al. 2005; Kim et al. 2006). Thus, PERK has protective functions, especially when first activated, but it can also induce cell death pathways if it is too strongly activated or active for too long.

12.2.3.2 PERK as Protective Mechanism in MM

As previously referenced, Michallet et al. used RNAi to individually knock down IRE1, ATF6, and PERK expression (Michallet et al. 2011). They observed that loss of any one sensor tended to increase the activation read outs of the remaining sensors, confirming crosstalk between the systems. Specific knockdown of PERK yielded two important findings. First, this single change resulted in an autophagic cell death response, implicating PERK activation as a necessary part of the metabolic shift from plasma cell to myeloma cell. Second, the loss of PERK impeded the apoptotic response. Therefore, PERK activity was implicated in both viability of myeloma cells and in the apoptotic potential of the cells (Michallet et al. 2011). This complex finding may shed light on idea of PERK activity as a potential danger to the cell.

12.2.3.3 PERK as a Cell Death Effector in MM

Activation of PERK has been implicated in a wide variety of cancers as a mediator of response to chemotherapy (Fribley et al. 2011; Kraus et al. 2008; Lust et al. 2009; Qiao et al. 2012; Sailaja et al. 2013; Yan et al. 2010). Most convincingly, siRNA against PERK or dominant negative models can ameliorate chemotherapy-induced death in many types of cancer cells (Kahali et al. 2010; Lai and Wong 2008; Pan et al. 2012; Yacoub et al. 2008). It is therefore perhaps unsurprising that this effect has also been seen in myeloma cells, which already have baseline ER stress and may not be able to tolerate perturbations to the system. In particular, researchers have been interested in the role of PERK in myeloma cell response to the proteasome inhibitor bortezomib, the most effective myeloma therapy. Studies have demonstrated that bortezomib treatment upregulates PERK activity as measured by ATF4 and downstream CHOP expression (Obeng et al. 2006). Furthermore, they correlated ER stress to bortezomib response by measuring the retention of immunoglobulin protein accumulating in treated cells. Myeloma cells that retained more of their secretory protein load, one hallmark of ER stress, showed more activation of ER stress markers and more sensitivity to the drug (Obeng et al. 2006).

This pathway has been further probed in myeloma cells by induction of ER stress through inhibition of heat shock proteins, the family of ER chaperones that includes both grp78 and gp96. Most commonly, heat shock protein 90 is targeted experimentally with the drug 17-AAD. By comparison of 17-AAG and bortezomib effects on myeloma cells, the study showed that both drugs induce upregulation of grp78, gp96, and CHOP, all of which are downstream effects of PERK activation

(Davenport et al. 2007). These effects were ultimately joined by an apoptotic response, suggesting that PERK activation culminated in a cell death program (Davenport et al. 2007).

The key component of apoptosis-induction by PERK was investigated to better understand the unfortunate phenomenon of bortezomib resistance in myeloma. Studies demonstrated that the phosphorylation of eIF2α is an indispensable aspect of PERK-mediated apoptosis (Schewe and Aguirre-Ghiso 2009). They studied a bortezomib-resistant subpopulation of myeloma cells and found that resistance could be reversed by inhibition of the eIF2α phosphatase or by competitive inhibition of the phosphatase via overexpression of a mutant phosphorylated eIF2α. In both conditions, cells with experimentally enhanced levels of endogenous phosphorylated eIF2α regained sensitivity to bortezomib (Schewe and Aguirre-Ghiso 2009).

The global repression of protein translation has far-reaching consequences, even if subsets of mRNAs are selectively processed. For instance, the balance of proteins in the cell quickly changes as proteins with short half-lives are degraded but not replaced. One system affected by such a change is the anti-apoptotic network, comprised of such anti-apoptotic proteins as survivin, Mcl-1, and FLIP, all of which are eliminated from the protein pool if not continuously generated (White-Gilbertson et al. 2009). This time-dependent shift in cellular fitness may be another axis on which PERK activation is titrated, so that short term activation is beneficial while long-term activation is ultimately detrimental to the cell.

12.3 Targeting UPR Pathways and HSPs Against MM: The Opportunities

UPR plays critical roles in plasma cell differentiation as well as in the pathogenesis of multiple myeloma (MM). XBP1s and downstream ER chaperones are consistently up regulated in myeloma cells in patients (Bagratuni et al. 2010), inspiring increasing efforts to develop UPR-targeted anti-MM therapy (Mimura et al. 2012; Papandreou et al. 2011; Ri et al. 2012).

12.3.1 Blockade of IRE1/XBP1s in MM

In addition to the critical roles of IRE1/XBP1 in plasma cell differentiation, a picture has emerged for the roles of UPR in myeloma. Indeed, XBP1s and downstream ER chaperones are consistently up regulated in myeloma patients. Patients with a low XBP1 spliced/unspliced ratio (≤1.33) have a longer overall survival compared with those with a higher ratio ($p = 0.03$, median, 56 months vs 40 months; HR = 1.75; 95 % CI = 1.07–2.85) (Bagratuni et al. 2010). Moreover, transgenic expression of XBP1s in mice also leads to plasma cell dyscrasia with evidence of increased

monoclonal antibodies ("M-spike"), lytic bone lesions, plasmacytosis and kidney damage (Carrasco et al. 2007). Given this information, IRE1/XBP1 could be a potential therapeutic target for multiple myeloma.

To investigate whether blocking the IRE1/XBP1 pathway is a therapeutic for MM, researchers performed chemical library screening and identified a small-molecule compound, STF-083010, which specifically blocks the endonuclease activity of IRE1 without affecting its kinase activity (Papandreou et al. 2011). Furthermore, treatment of a variety of myeloma cell lines with STF-083010 in vitro demonstrated its potent activity against myeloma. Importantly, STF-083010 is also selectively more cytotoxic to freshly isolated $CD138^+$ plasma cells from myeloma patients than $CD19^+$ B cells, $CD3^+$ T cells and $CD56^+$ NK cells. Finally, treatment of human myeloma xenografts in NSG mice was performed. STF-083010 was given by intraperitoneal injection on day 1 and day 8 and this compound significantly inhibited the growth of these tumors in vivo (Papandreou et al. 2011). In addition, another small-molecule inhibitor, MKC-3946 also blocks the IER1α endoribonuclease domain. MKC-3946 inhibits multiple human myeloma cell lines without toxicity to normal mononuclear cells. MKC-3946 also blocks ER stress induced by both bortezomib and heat shock protein 90 inhibitor 17-AAG. Moreover, MKC-3946 can significantly enhance the cytotoxicity of MM cells induced by bortezomib or 17-AAG (Mimura et al. 2012). A similar result was found by using an XBP1 inhibitor, toyocamycin, which was identified from the culture broth of an *Actinomycete* strain. Toyocamycin has been shown to suppress the *XBP1* mRNA splicing in HeLa cells which is induced by thapsigargin, tunicamycin, and 2-deoxyglucose. It does not, however, affect ATF6 and PERK activation. Although toyocamycin does not inhibit IRE1α phosphorylation, it prevents IRE1α-induced *XBP1* mRNA cleavage and inhibits constitutive activation of XBP1 expression in myeloma cell lines as well as in samples from myeloma patients in vitro. Toyocamycin also induces apoptosis of myeloma cells, including bortezomib-resistant myeloma cells, and it inhibits myeloma cell growth in a human myeloma xenograft model (Ri et al. 2012). Taken together, these results demonstrate that blockade of IRE1/XBP1 pathway by small-molecule compounds is a potentially useful therapeutic modality for human myeloma (Table 12.1).

12.3.2 Targeting HSPs Is a Promising Therapeutic Platform for MM

Heat shock proteins are a group of highly conserved proteins, which are involved in many cellular processes such as protein folding, intracellular trafficking, modulating signaling pathways and regulating immune responses. Heat shock proteins play critical roles in the regulation of protein homeostasis and cell survival. Inhibition of HSPs results in a disruption of protein processing and induces ER stress. Therefore, HSPs are attractive targets for treatment of MM (Table 12.2).

Table 12.1 Summary of targeting of UPR pathways in MM

Agent	Target	Effect on UPR and MM	Phase	References
Toyocamycin	IRE1α-XBP1	Induce XBP1s, not affect ATF6 and PERK	Preclinical studies	Ri et al. (2012)
		Induce apoptosis of MM cells, including bortezomib-resistant cells in vitro, and also inhibit MM cell growth in vivo		
STF-083010	IRE1α	Block the endonuclease activity without affecting kinase activity	Preclinical studies	Papandreou et al. (2011)
		Inhibit MM cell growth in vitro and in vivo		
MKC-3946	IRE1α	Block the endonuclease activity	Preclinical studies	Mimura et al. (2012)
		Inhibit MM cell growth in vitro and in vivo. Blocks ER stress induced by bortezomib and HSP90 inhibitor 17-AAG		

12.3.2.1 Targeting HSP90 in MM

Cytosolic HSP90 is a key molecular chaperone machinery. Up to 10 % of cytosolic proteins are client proteins of HSP90 (McClellan et al. 2007; Zhao et al. 2005), many of which are critical for cell survival and proliferation. The HSP90 chaperone is regulated by a conserved ATP-binding pocket located at the N-terminal domain. While bound to HSP90s, the regulatory nucleotides, ATP/ADP, adopt a unique bent shape (Chene 2002). Such a distinct pocket is especially useful for drug discovery for being different from those of other ATPases. Several HSP90 inhibitors are currently being tested in early phase clinical trials (Chandarlapaty et al. 2008; Kummar et al. 2010; Richardson et al. 2010; Solit et al. 2008). Geldanamycin and its analogue, 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibit the proliferation of MM cell lines in part via ER stress and UPR death pathway. IPI-504 is a highly soluble HSP90 inhibitor and induces apoptosis of MM cells, which is mediated by inactivating XBP1 and ATF6 (Patterson et al. 2008). Tanespimycin (17-allylamino-17-demethoxygeldanamycin, 17-AAG) is a synthetic geldanamycin analogue, which induces apoptosis in both drug-sensitive and drug-resistant MM cell lines as well as in MM cells from relapsed MM patients (Mitsiades et al. 2006). The phase I clinical trial of Tanespimycin was completed (Richardson et al. 2010). In addition, another highly selective small molecule inhibitor of HSP90, PF-04929113 (SNX-5422) has entered into early phase human clinical trials (Reddy et al. 2013). One patient with multiple myeloma had prolonged stabilization of disease for at least 20 months before disease progression. These studies reveal that the disrupting HSP90 chaperone activity results in a complex modulation of ER stress, UPR and cell death pathways. The field is waiting anxiously for the clinical experience of HSP90 inhibitors in more advanced stage of clinical testing (Li et al. 2011; Usmani et al. 2009).

Table 12.2 Summary of targeting of HSPs in MM

Agent	Target	Effect on UPR and MM	Phase	References
SNX-2112	HSP90	Induce apoptosis via caspase 8 and 9 and inhibit Akt and extracellular signal-related kinase activation	Preclinical studies	Okawa et al. (2009)
		Inhibited MM growth and prolong survival in a xenograft murine model		
IPI-504	HSP90	Inactivate XBP1 and ATF6	Preclinical studies	Patterson et al. (2008)
		Induce MM cell apoptosis in vitro		
Tanespimycin	HSP90	A minimal therapeutic response in relapsed and refractory MM patients	I	Mitsiades et al. (2006) and Richardson et al. (2010)
PF-04929113	HSP90	A prodrug of SNX-2112	I	Reddy et al. (2013)
		Prolong stabilization of disease on one MM patient		
PU-WS13	gp96	Not induce UPR	Preclinical studies	Hua et al. (2013)
		Induce apoptosis and inhibit growth of multiple MM cells in vitro		
Ver-155008	HSP70	Induced apoptosis of multiple MM cells and enhanced HSP90 inhibition induced cell death	Preclinical studies	Chatterjee et al. (2013) and Zhang et al. (2013)
MAL3-101	HSP40/ HSP70	Inhibit proliferation and survival of primary MM cells and endothelial progenitor cells obtained from MM patients	Preclinical studies	Braunstein et al. (2011)

12.3.2.2 Targeting ER Chaperone gp96 in MM

gp96 (Srivastava et al. 1986), also known as grp94 (Lee et al. 1981), endoplasmin (Koch et al. 1986), ERp99 (Lewis et al. 1985), and HSP90b1 (Chen et al. 2005); is an ER paralogue of HSP90. Like other HSPs, gp96 is induced by the accumulation of misfolded proteins (Kozutsumi et al. 1988). It binds to and hydrolyzes ATP (Dollins et al. 2007; Frey et al. 2007; Li and Srivastava 1993), it is the most abundant and a ubiquitous protein in the ER lumen. gp96 is a key downstream chaperone in the ER and mediates UPR. Our recent study demonstrated that gp96 is required for the development of MM during chronic ER stress conditions (Hua et al. 2013). We selectively deleted gp96 in B cells in XBP1s transgenic mice which overexpress XBP1s in B cells and plasma cells and spontaneously develop multiple myeloma after reaching advanced age (Carrasco et al. 2007). We found that gp96 is required for maintenance of plasma cells in this model and it is a key driver for development of

MM. Moreover, we demonstrated both genetically and pharmacologically that targeted inhibition of gp96 resulted in significant compromise in MM cell growth and massive apoptosis. PU-WS13, a gp96-specific HSP90 inhibitor of the purine-scaffold class (Taldone and Chiosis 2009) was tested against MM cells. We found that PU-WS13 selectively induced apoptosis and inhibited growth of multiple MM cells, which is in part due to the loss of canonical Wnt pathway (Hua et al. 2013; Liu et al. 2013). Thus, further development of gp96-targeted inhibitors such as PU-WS13 in MM is warranted.

12.3.2.3 Targeting HSP70 in MM

HSP70 family proteins are located in various subcellular locations, which include the constitutively expressed HSC70 and stress induced HSP72 in the cytoplasm, grp78 in the ER and mortalin/Grp75 in the mitochondria. HSP70 forms a complex with its co-chaperone Hsp40 to assist protein folding and maintain protein homeostasis and cell survival (Mayer et al. 2000). With the complex, the HSP70 is involved in cell cycle regulation, apoptosis and differentiation. In addition, the cytoplasmic inducible HSP72 and its cognate protein HSC70 are responsible for protein folding in the cytoplasm as well as the recruitment of E3 ubiquitin ligases such as CHIP to tag target proteins for proteasomal degradation (Park et al. 2007). In addition to their roles in maintaining the cellular protein homeostasis, cytoplasmic HSP70 inhibits both the caspase dependent and independent apoptosis pathways (Sabirzhanov et al. 2012). Base on the functions of HSP70, HSP70 inhibitors would be potentially useful for the treatment of MM. Currently, only two HSP70 specific small compounds, Ver-155008 and MAL3-101, have been tested for MM in the preclinical setting. Ver-155008 is an ATP-analogue. Treatment with VER-155008 induced apoptosis of multiple MM cells, resulting in decreased levels of HSP90 clients affecting multiple oncogenic signaling pathways (Chatterjee et al. 2013; Zhang et al. 2013). In contrast to Ver-155008, MAL3-101 inhibits the ability of Hsp40 co-chaperone to stimulate HSP70 ATPase activity, thereby blocking HSP70 functions (Fewell et al. 2004). MAL3-101 exhibited promising anti-myeloma properties against myeloma cell lines in vitro and in vivo, and demonstrated synergy with proteasome and HSP90 inhibitors (Braunstein et al. 2011). These studies provide evidence that targeting HSP70 is a promising approach for MM.

12.4 Conclusions and Perspectives

The integrated ER stress response is composed of all three sensor systems and their interplay determines the overall cellular strategy and the outcome of stress. The evidence for the importance of UPR in MM is mounting (White-Gilbertson et al. 2013). Both genetic and chemical tools have now been used to tease out the contribution of each branch of UPRs and their downstream effector molecules in

MM. Collectively, myeloma cells appear to harbor an Achilles heel in their baseline metabolism and dependence on UPR, as shown by a uniformed death outcome after perturbation of multiple sensors and HSPs in the ER stress response. This metabolic addiction to pathways that prevent UPR-induced death program may be a key target for MM treatment, which deserves more focused attention. One example of such effort is the possibility of PERK inhibitors as cancer therapeutics (Bi et al. 2005; Hart et al. 2012). It is also possible that a unique adaptive UPR program is adopted by individual myeloma patients, having diseases with different vulnerabilities. An individualized strategy with an array of tools to inhibit or push ER stress may be needed to overcome the adaptive nature of MM in protein quality control to reach a therapeutic benefit.

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Chapter 13
Melanomagenic Gene Alterations Viewed from a Redox Perspective: Molecular Mechanisms and Therapeutic Opportunities

Georg T. Wondrak

Abstract The causative involvement of altered redox homeostasis and reactive oxygen species (ROS)-dependent signaling in the control of survival, proliferation, and invasiveness of cancer cells has recently emerged. A large body of experimental and epidemiological research has substantiated the causative involvement of specific genetic alterations in melanomagenesis. Strikingly, some of the proteins encoded by specific genes underlying melanomagenesis (*CDKN2A*, *MC1R*, *MITF*, *KIT*, *NRAS*, *BRAF*, *AKT3*, *PTEN*, *RAC1*, *MAP3K5*, *KEAP1*, *MYC*) assume mechanistic roles in the control of cellular redox signaling and oxidative stress, thereby fulfilling molecular functions relevant to suppression or promotion of tumorigenesis that reach beyond their canonical activities, a significant yet underappreciated phenomenon that may open avenues towards novel redox-directed chemotherapeutic interventions as discussed in this chapter.

Keywords Reactive oxygen species • Redox dysregulation • Oxidative stress • *CDKN2A* • *MC1R* • *MITF* • *KIT* • *NRAS* • *BRAF* • *AKT3* • *PTEN* • *RAC1* • *MAP3K5* • *KEAP1* • *MYC* • Melanomagenesis • Melanoma • Melanocyte • Synthetic lethality • Targeted therapeutics • Chemotherapy

13.1 Melanoma Skin Cancer: The Emergence of Molecularly Targeted Therapeutics

The term melanoma refers to a group of melanocytic malignancies originating from neural crest-derived melanocytes that causes the majority of skin cancer-related deaths worldwide, representing a public health burden of considerable magnitude

G.T. Wondrak (✉)
Department of Pharmacology and Toxicology, College of Pharmacy and The University of Arizona Cancer Center, University of Arizona, 1515 N. Campbell Ave, Tucson, AZ 85724, USA
e-mail: wondrak@pharmacy.arizona.edu

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(Ibrahim and Haluska 2009; Luke and Hodi 2013). Remarkably, specific melanoma subtypes are characterized by distinct genetic fingerprints (Tsao et al. 2012; Kunz 2014). For example, superficial spreading melanoma and nodular melanoma, common forms of cutaneous melanoma, are associated with *BRAF* or *NRAS* mutations. In contrast, acral lentiginous melanoma is associated with *KIT* alterations, and ocular melanoma (that does not display *BRAF*, *NRAS*, or *KIT* changes) is driven by alterations of *GNA11* or *GNAQ*. The successful development of therapies that target mutated kinases such as BRAF or c-KIT has resulted in new genotype-directed personalized treatment options including vemurafenib, dabrafenib, trametinib, imatinib, and other kinase inhibitors (Tsao et al. 2012; Luke and Hodi 2013; Karimkhani et al. 2014).

The development of molecularly targeted small molecule therapeutics has recently revolutionized melanoma chemotherapy. Both dacarbazine, representing the standard of care for more than three decades after its initial FDA-approval in 1975, as well as high-dose interleukin-2, approved by the FDA in 1998, benefit only small subsets of melanoma patients and have now been surpassed by molecularly targeted agents with improved clinical efficacy (Yang et al. 2010). The mitogen-activated protein (MAP) kinase pathway presents multiple opportunities for therapeutic intervention, and due to its crucial involvement in melanomagenesis $BRAF^{V600E}$ has become a molecular target for the development of ATP-competitive inhibitors as a novel class of melanoma chemotherapeutics, complemented by a companion diagnostic assessing $BRAF^{V600E}$ mutational status. Currently, the BRAF inhibitors vemurafenib and dabrafenib and the MEK inhibitor trametinib have demonstrated significant clinical benefit and have been FDA approved for use in patients with BRAF mutations, achieving considerable clinical response rates but only moderate effects on median progression-free survival (less than 1 year) (Chapman et al. 2011). In a recent randomised, open-label clinical trial assessing safety and efficacy of vemurafenib, significant improvement in median overall survival (13.6 months) and median progression-free survival (6.9 months) was achieved in patients with either $BRAF^{V600E}$ or $BRAF^{V600K}$ mutation-positive melanoma (McArthur et al. 2014). Resistance to BRAF inhibitors represents a formidable clinical challenge. Multiple mechanisms of resistance to BRAF inhibitors are operative, including expression of $BRAF^{V600E}$ splice variants that lack the RAS-binding domain yet retain BRAF kinase activity in the presence of vemurafenib, a property attributed to enhanced homodimerization (Basile et al. 2014). In addition, other resistance mechanisms cause reactivation of ERK1/2 signaling, including *BRAF* amplification, upregulation of receptor tyrosine kinases such as PDGFRβ and IGF-1R, overexpression of *MAP3K8*, mutation of RAS isoforms, and mutations in *MEK1* and *MEK2*.

In addition to small molecule therapeutics that modulate aberrant oncogenic signaling pathways, the development of antibody-based biologicals that target critical components of T cell regulatory pathways has contributed to the recent paradigm shift in melanoma pharmacotherapy (Karimkhani et al. 2014). T cell inactivation is a crucial factor preventing immune dysregulation and autoimmunity, but it also represents an important mechanism of immune evasion characteristic of malignant melanoma. One process of inactivation involves the expression of the CTLA-4T cell

surface receptor that initiates inhibitory signaling suppressing T cell function upon binding to B7 on antigen presenting cells, a regulatory process operative in the early phase of T cell activation. Another process resulting in T cell inactivation operative during later stages involves the expression of the PD-1 (programmed cell death-1) receptor on the T cell surface, which then binds to PD-L1 (programmed cell death 1 ligand 1) on tumor tissue. Inhibition (or checkpoint blockade) of CTLA-4 or PD-1 (or PD-L1) can therefore promote anti-tumour immunity. CTLA4- (ipilimumab and tremelimumab), PD-1- (nivolumab, lambrolizumab, pidilizumab), and PD-L1-directed (BMS-936559) antibody-based biologicals are now in advanced clinical studies or have already received FDA approval, and phase I safety studies of anti-LAG-3 (lymphocyte-activation gene 3; BMS-986016) with and without anti-PD-1 (nivolumab) in the treatment of solid tumors are ongoing.

Importantly, despite recent progress in the design of targeted therapies, efficacy of therapeutic intervention remains limited with only moderate effects on median progression-free survival (measured in months) associated with these non-curative interventions that are compromised by the rapid development of drug resistance (Chapman et al. 2011). Consequently, an urgent need exists for the identification and development of improved molecular agents that target specific molecular vulnerabilities of metastatic melanoma cells (Hoeflich et al. 2006; Ibrahim and Haluska 2009; Yang et al. 2010; Aplin et al. 2011; Chapman et al. 2011; Kudchadkar et al. 2012; Luke and Hodi 2013).

13.2 Redox-Directed Cancer Therapeutics

The causative involvement of altered redox homeostasis and reactive oxygen species (ROS)-dependent signaling in the control of survival, proliferation, and invasiveness of cancer cells has recently been substantiated. Importantly, cumulative experimental evidence suggests that redox dysregulation originating from metabolic alterations and dependence on mitogenic and survival signaling through reactive oxygen species represents a specific vulnerability of malignant cells that can be selectively targeted by redox chemotherapeutics (Trachootham et al. 2009; Wondrak 2009; Gorrini et al. 2013). Feasibility of redox-directed intervention for the targeted chemotherapeutic induction of cancer cell apoptosis has been explored earlier based on the rational that small molecule pro-oxidant intervention may cause cytotoxic deviations from redox homeostasis that induce apoptosis in malignant cells, already exposed to high constitutive levels of ROS, without compromising viability of non-transformed cells.

Importantly, it has been shown that the pleiotropic action of many redox chemotherapeutics that involves simultaneous modulation of multiple redox sensitive targets can overcome cancer cell drug resistance originating from redundancy of oncogenic signaling and rapid mutation. Indeed, numerous preclinical and clinical studies have explored the tumor-directed efficacy of experimental and investigational

redox chemotherapeutics as reviewed recently (Trachootham et al. 2009; Wondrak 2009; Gorrini et al. 2013; Nogueira and Hay 2013). The impressive number of ongoing clinical trials that examine therapeutic performance of novel redox drugs in cancer patients demonstrates that redox chemotherapy has made the crucial transition from bench to bedside.

The emerging causative link between melanoma and altered redox homeostasis has prompted preclinical and clinical examinations employing redox agents including the glutathione biosynthesis inhibitor L-buthionine sulfoximine, the glutathione-depleting agent imexon, and the proxidants disulfiram, ATN-224, and elesclomol, but none of these interventions has moved successfully beyond the stage of phase III clinical testing (Fruehauf and Trapp 2008; Trapp et al. 2009; Weber et al. 2010; O'Day et al. 2013). Our own preclinical prototype studies have documented the feasibility of redox intervention targeting malignant melanoma cells through small molecule pro-oxidant therapeutics (Wondrak 2007, 2009; Cabello et al. 2009, 2012; Qiao et al. 2012a, b). However, as of today, no melanoma-directed redox chemotherapeutics are available for clinical use.

13.3 Redox Dysregulation in Melanomagenesis

A causative involvement of altered redox homeostasis mediated through reactive oxygen species (ROS) in melanomagenesis has recently been substantiated as expertly reviewed (Meyskens et al. 1999; Cheng et al. 2004; Govindarajan et al. 2007; Wittgen and van Kempen 2007; Fried and Arbiser 2008; Fruehauf and Trapp 2008; Jenkins et al. 2011; Denat et al. 2014; Liu-Smith et al. 2014). It has been demonstrated that epidermal melanocytes are particularly vulnerable to oxidative stress originating in part from melanin biosynthesis that may occur downstream of solar UV exposure or postinflammatory hyperpigmentation. Formation of redox-active pathological melanins, melanosomal leakage of pro-oxidant melanin precursors, and transition metal ion dysregulation all have been implicated as causative factors underlying redox alterations that contribute to melanomagenesis, and increased endogenous oxidative stress has been associated with disease progression (Meierjohann 2014). Befittingly, melanocytes have been referred to as 'instigators and victims of oxidative stress' (Denat et al. 2014). Moreover, the redox-directed function of key regulators of oxidative stress in melanomagenesis has recently been explored including the pro-oxidant activity of NAD(P)H oxidases and mitochondrial respiratory activity as discussed below (Brar et al. 2001; Govindarajan et al. 2007; Yamaura et al. 2009; Graham et al. 2010; West et al. 2010; Jenkins et al. 2011; Liu-Smith et al. 2014; Theodosakis et al. 2014). Melanoma has been labeled 'the reactive oxygen-driven tumor' (Fried and Arbiser 2008). Indeed, cumulative research suggests a causative involvement of endogenous production of ROS and redox dysregulation in the control of melanoma cell survival, proliferation, and invasiveness mediated through redox-sensitive targets including components of signaling cascades and transcription factors (such as e.g. PTEN, AKT, NRF2, NFκB,

AP-1, APE-Ref1, and HIF1α), and it has been proposed that specific molecular vulnerabilities that result from redox dysregulation represent a molecular Achilles heel that can be targeted by specific redox intervention (Meyskens et al. 2001; Wittgen and van Kempen 2007; Fried and Arbiser 2008; Fruehauf and Trapp 2008; Meierjohann 2014).

13.4 Melanomagenic Gene Alterations Viewed from a Redox Perspective

A large body of experimental and epidemiological research has substantiated the causative involvement of specific genetic alterations in melanomagenesis. Recent high-throughput whole-genome sequencing efforts have greatly expanded the number of known hereditary or somatic genetic alterations involved in determining disease susceptibility and driving melanoma initiation and progression (Hodis et al. 2012; Tsao et al. 2012; Kunz 2014). A wide array of alterations has been documented impacting genes encoding kinases (*BRAF*, *KIT*, *ERBB4*, *AKT3*, *PIK3CA*, *MAP3K5*, *MAP3K9*, *CDK4*) and kinase inhibitors (*CDKN2A*), GTPases (*NRAS*, *RAC1*, *GNAQ*, *GNA11*), phosphatases (*PTEN*), GTPase and phosphatase regulatory factors (*PREX2*), transcription factors (*MITF*, *MYC*), receptors for glutamate (*GRIN2A*, *GRM3*) or melanocortin (*MC1R*), proteases (*MMP8*), and modulators of proteasome-dependent substrate degradation (*KEAP1*, *CDKN2A*). Strikingly, some of the proteins encoded by these genes assume mechanistic roles in the control of cellular redox homeostasis and redox signaling or can themselves be subject to redox modulation, thereby fulfilling molecular functions relevant to suppression or promotion of tumorigenesis that reach beyond their canonical activities, a significant yet underappreciated phenomenon that may open avenues towards novel therapeutic interventions as discussed in the following paragraphs (Fig. 13.1).

13.4.1 MC1R and MITF in Melanomagenesis and Redox Modulation

MC1R encodes a cyclic AMP-stimulating α_s-type G protein-coupled receptor that controls pigment production through CREB (cAMP response element-binding protein)-dependent upregulation of *MITF* (encoding microphthalmia-associated transcription factor), a basic helix-loop-helix leucine zipper transcription factor that serves as the master regulator of melanocyte differentiation controlling the transcription of melanogenic genes including *TYR*, *TYRP1*, and *DCT* (Tsao et al. 2012; Denat et al. 2014). During solar UV-induced tanning, MC1R is activated by melanocortins including α-MSH (α-melanocyte stimulating hormone) producing eumelanin, whereas impaired receptor function results in pheomelanin production.

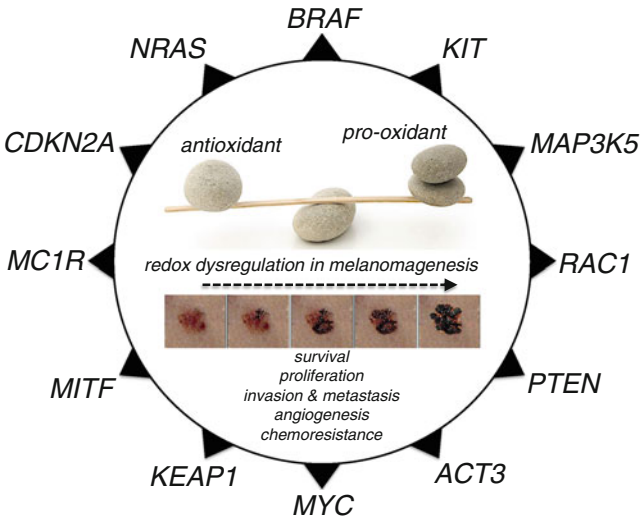

Fig. 13.1 Redox dysregulation in melanomagenesis driven by genetic alterations. Cumulative experimental evidence indicates that melanoma progression (from benign nevi to the metastatic stage of the disease) is modulated by redox dysregulation. Alteration of specific genes with an established causative role in melanomagenesis may also impact cellular redox homeostasis and signaling, contributing to suppression or promotion of tumorigenesis and opening novel avenues for redox-directed therapeutic interventions

Importantly, *MITF* has been proposed to act as an oncogene in melanomagenesis stimulating the transcription of hypoxia inducible factor (*HIF1A*) with upregulation of VEGF expression, suggesting the existence of an α-MSH/MC1R-cAMP-CREB-MITF-HIF1α axis that contributes to melanoma progression (Busca et al. 2005). Moreover, *MITF* might play a causative role in conferring a genetic susceptibility to co-occurring melanoma and renal cell carcinoma based on the finding that a SUMOylation-defective *MITF* germline mutation predisposes to melanoma and renal carcinoma (Bertolotto et al. 2011). *MITF* repression by posttranslational SUMOylation is of fundamental importance since germline activational *MITF* gene mutations (Mi-E318K) inhibiting SUMOylation occur at a significantly higher frequency in genetically enriched patients affected with melanoma, renal cell carcinoma or both cancers when compared to controls. In addition, Mi-E318K enhanced MITF protein binding to the *HIF1A* promoter and increased its transcriptional activity compared to wild-type MITF.

Intriguingly, cumulative evidence suggests an important role of MITF in the control of redox homeostasis in melanocytes and melanoma cells. First, it has recently been shown that oncogenic BRAF regulates mitochondrial respiration and oxidative metabolism via PGC1α and *MITF* (Vazquez et al. 2013). Moreover, MITF regulates melanoma cell response to oxidative stress through transcriptional regulation of APE-1/Ref-1 [apurinic/apyrimidinic endonuclease/redox effector-1)], a key redox regulator and DNA endonuclease involved in repair of oxidative DNA damage

(Liu et al. 2009). In addition, MITF has been shown to increase gene expression of NADPH oxidase type 4, a negative redox-regulator of melanogenesis (Liu et al. 2012b).

MC1R is highly polymorphic displaying over 60 variants in Caucasian skin, and germline variants of *MC1R* that disrupt the cAMP-MITF signaling cascade are present in 80 % of red haired individuals. It is now widely accepted that gene polymorphisms affecting *MC1R* that underlie pheomelanogenesis and impaired solar tanning represent predictive markers of melanoma susceptibility (Tsao et al. 2012). In addition, the *MC1R* genotype is an established determinant of the damage response of melanocytes to ultraviolet radiation, and MC1R signaling has now been recognized as a major regulator of redox homeostasis in melanocytes, acting in part through upregulation of antioxidant pathways that limit UV-induced photo-oxidative DNA damage with enhancement of OGG1-dependent repair of oxidative DNA base lesions (Kadekaro et al. 2012). Importantly, α-MSH-MC1R signaling controls intracellular redox status through control of antioxidant gene expression (e.g. *HMOX1*, *GCLC*, *PRDX1*) downstream of multiple redox-directed transcriptional modulators (MITF, APE-1, NRF2), and cells expressing dysfunctional *MC1R* variants display higher levels of constitutive oxidative stress, potentially contributing to ROS-induced BRAF mutations (Tsao et al. 2012; Marrot et al. 2008; Kadekaro et al. 2012; Denat et al. 2014). As an additional mechanism of action, pheomelanin precursors have been identified as potent redox-active sensitizers of UVA-induced photooxidative stress, and UVA-induced but not UVB-induced murine melanomagenesis has recently been shown to occur as a function of melanogenesis associated with oxidative DNA damage in melanocytes (Noonan et al. 2012). Strikingly, it has also been demonstrated that pheomelanogenesis contributes to solar UV-independent melanomagenesis thought to originate from the causation of melanogenesis-associated oxidative damage. Selective absence of pheomelanin synthesis reduced oxidative DNA and lipid peroxidation damage and was protective against melanoma development, whereas $BRAF^{V600E}$ expression in a pheomelanin-based 'redhead' mouse model with inactive MC1R led to an increased risk of invasive melanoma (Mitra et al. 2012).

13.4.2 CDKN2A in Melanomagenesis and Redox Modulation

The *CDKN2A* (cyclin-dependent kinase N2A) gene encoding the tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$ has been identified as a crucial melanoma susceptibility factor, representing a critical target of inactivation (i.e. loss of function) at both the germline and somatic levels (Tsao et al. 2012). In familial cutaneous malignant melanoma, accounting for ~10 % of all cutaneous malignant melanoma cases, the most common known high-penetrance susceptibility gene is *CDKN2A* (followed by *CDK4*). The $p16^{INK4A}$ cyclin-dependent kinase inhibitor exhibits a cell cycle regulatory function by binding to CDK4/6 leading to a reduction in Rb phosphorylation and inhibition of the G_1/S transition. The alternate reading frame product $p14^{ARF}$

interacts and antagonizes HDM2 (human double minute 2 homolog), an E3 ubiquitin-protein ligase that targets the tumor suppressor p53 towards proteasomal degradation. Homozygous deletion of *CDKN2A* deletes both $p16^{INK4A}$ and $p14^{ARF}$, compromising the $p16^{INK4A}$-CDK4-Rb and $p14^{ARF}$-HDM2-p53 tumor suppressor pathways, and *CDKN2A* loss renders p53 mutation redundant, a finding consistent with the observation that the p53 mutation rate is low in melanoma. Inactivation of the $p16^{INK4A}$ gene through germ-line mutations is associated with hereditary melanoma predisposition, and most *CDKN2A* germline mutations that confer melanoma risk occur in exons 1α and 2 (encoding portions of $p16^{INK4a}$) suggesting that $p16^{IN4a}$ is the preferentially targeted and functionally dominant component of *CDKN2A*.

Recently, a novel and unexpected role of $p16^{INK4A}$ in redox regulation of melanomagenesis has emerged (Jenkins et al. 2011, 2013). In human melanocytes, $p16^{INK4A}$ has been identified as a redox responsive factor, undergoing rapid upregulation following solar UV- or hydrogen peroxide-dependent p38 MAPK signaling. Strikingly, genetic antagonism of $p16^{INK4A}$ increases oxidative stress and oxidative DNA damage in melanocytes suggesting that $p16^{INK4A}$ plays a heretofore unrecognized role as a cytoprotective redox modulator in melanocytes. The increased susceptibility of melanocytes to oxidative stress as a result of $p16^{INK4A}$ depletion suggests a dual tumor suppressor function of $p16^{INK4A}$, preventing oncogenic oxidative DNA lesions by controlling oxidative stress on one hand and allowing DNA repair after induction of cell cycle arrest through the $p16^{INK4A}$-CDK4-Rb pathway on the other. More recently, it has been demonstrated that specific familial melanoma-associated $p16^{INK4A}$ mutations can selectively compromise either one of these two tumor suppressor functions mediated by distinct regions of the protein. Indeed, familial melanoma-associated point mutants spanning the $p16^{INK4A}$ coding region displayed differential effects on cell cycle regulation and modulation of oxidative stress, with several mutations impairing cell cycle (R24Q, R99P, V126D) or redox functions (A36P, A57V, P114S) selectively, a finding indicative of cell cycle- and redox-directed activities of $p16^{INK4A}$ that can be mutually uncoupled in human melanoma cells.

Redox modulation exerted by various tumor suppressor genes represents a molecular function that reaches beyond their canonical activities and has attracted considerable interest. Similar to an emerging antioxidant function of p16 suppressing melanomagenesis, the *CDKN1A*-encoded tumor suppressor p21 (CIP1/WAF1) has been shown to downregulate oxidative stress by increasing stability of NRF2 with upregulation of antioxidant NRF2-target gene expression (Chen et al. 2009). Indeed, other tumor suppressor genes including *TP53*, the upstream regulator of *CDKN1A*, and *BRCA1* are known to control oxidative DNA base lesions and redox homeostasis through upregulation of various redox-directed target genes as reviewed recently (Vurusaner et al. 2012).

13.4.3 NRAS and BRAF in Melanomagenesis and Redox Modulation

Activation of the ERK-mitogen activated protein kinase (MAPK) pathway due to oncogenic mutation of *NRAS* and *BRAF* is a primary mechanistic driver in melanomagenesis, and activating mutations in the *NRAS* and *BRAF* oncogenes, which

are both mutually exclusive, have been identified in a majority of melanomas (Davies et al. 2002; Tsao et al. 2012; Kunz 2014). Activating mutations in the *NRAS* small GTPase are present in 15–20 % of all melanomas with the majority of mutations represented by $NRAS^{Q61R}$ or $NRAS^{Q61K}$. In addition to the MAPK pathway, *NRAS* simultaneously activates the phosphatidyl-inositol 3-kinase (PI3K) pathway. RAS-driven tumorigenesis has been the target of various stress response pathway-directed therapeutic strategies but no specific interventions targeting NRAS in melanoma have been reported (Yang and Stockwell 2008; De Raedt et al. 2011).

BRAF is a serine/threonine kinase involved in the Ras-Raf-MAPkinase pathway. Remarkably, activating mutations in *BRAF* are the most common genetic alterations in melanoma, and gain-of-function mutations of the *BRAF* gene are found in the vast majority of benign nevi and primary melanomas representing a key event in early melanomagenesis (Davies et al. 2002). Numerous *BRAF* mutations have been documented, the most frequent of which results in a valine-to-glutamic acid change at position 600 ($BRAF^{V600E}$) constitutively activating this protein kinase upstream of MEK and ERK1/2, which in turn phosphorylates transcription factors that control proliferative and anti-apoptotic targets. Although UV exposure is a major risk factor for melanoma, $BRAF^{V600E}$ originates from a T>A transversion, rather a common UV-associated C>T transition, an unexplained phenomenon that has been attributed to oxidative stress-induced mutagenesis downstream of melanogenesis-derived ROS formation (Tsao et al. 2012).

Importantly, downstream of $BRAF^{V600E}$ or $NRAS^{Q61R}$ expression, oncogene-induced senescence is a common occurrence that determines the senescent phenotype displayed by melanocytes in benign nevi. Indeed, benign melanocytic lesions harboring a mutant allele of *BRAF* or *NRAS* display crucial hallmarks of senescence including proliferative arrest, $p16^{INK4A}$ upregulation, and senescence-associated β-galactosidase expression. It has been demonstrated earlier that receptor tyrosine kinase- or $NRAS^{Q61K}$-signaling leads to redox dysregulation characterized by accumulation of high ROS levels in melanocytes, an oxidative stress-induced senescent phenotype suppressed by antioxidant supplementation (Leikam et al. 2008). Interestingly, the ROS-generating oxidases Nox1 and Nox4 are an important source of endogenous oxidative stress contributing to oncogenic Ras-induced premature senescence (Kodama et al. 2013). In addition, mitochondrial respiratory electron leakage might contribute to ROS-mediated oncogene-driven senescence, and oncogene-dependent regulation of mitochondrial function, oxidative metabolism, and redox regulation in melanoma have recently attracted considerable interest (Theodosakis et al. 2014). Importantly, the mitochondrial gatekeeper pyruvate dehydrogenase (PDH) has been identified as a crucial mediator of $BRAF^{V600E}$-induced oxidative stress-mediated senescence, accompanied by simultaneous suppression of the PDH-inhibitory enzyme pyruvate dehydrogenase kinase 1 (PDK1) and induction of the PDH-activating enzyme pyruvate dehydrogenase phosphatase 2 (PDP2) (Kaplon et al. 2013). Activation of PDH-enhanced pyruvate metabolism through the tricarboxylic acid cycle causes increased mitochondrial respiration and ROS formation, and engineered normalization of either PDK1 or PDP2 expression levels resulting in PDH inhibition enables $BRAF^{V600E}$-driven melanoma development by

abrogation of $BRAF^{V600E}$-induced senescence. The observation that depletion of PDK1 eradicated melanoma subpopulations resistant to targeted BRAF inhibition causing regression of established melanoma tumors suggests that therapeutic interventions upregulating pyruvate mitochondrial metabolism can be harnessed to antagonize tumor growth in BRAF-driven melanoma by reestablishing oncogene-induced senescence.

Recently, it has been reported that oncogenic BRAF regulates mitochondrial oxidative metabolism via the mitochondrial master regulator PGC1α and MITF (Vazquez et al. 2013). Melanomas displaying activation of the BRAF/MAPK pathway have suppressed levels of MITF (an established BRAF target) and PGC1α resulting in decreased oxidative mitochondrial metabolism. In contrast, BRAF inhibition induces an oxidative phosphorylation gene program and mitochondrial biogenesis together with increased expression of PGC1α, indicating that treatment of BRAF-mutated melanomas with BRAF inhibitors causes a functional dependence on oxidative phosphorylation. These data suggest an adaptive metabolic program that limits the efficacy of BRAF inhibitors, and mitochondrial uncouplers undermining oxidative phosphorylation may therefore have therapeutic use in combination with BRAF inhibitors (Haq et al. 2013).

In addition to studies that substantiate the vemurafenib-induced mitochondrial oxidative metabolism in $BRAF^{V600E}$ melanomas expressing PGC1α, mitochondrial oxidative stress has also been identified as a specific vulnerability characteristic of melanoma cells resistant to $Braf^{V600E}$ inhibitors (Corazao-Rozas et al. 2013). The observation that elevated ROS levels rendered vemurafenib-resistant melanoma cells prone to cell death induced by pro-oxidants including the investigational redox drug elesclomol suggests that the mitochondrial oxidative signature of resistant melanoma constitutes a druggable redox vulnerability, an intriguing hypothesis to be pursued by ongoing preclinical and clinical research.

13.4.4 KIT in Melanomagenesis and Redox Modulation

The *KIT* gene encodes the transmembrane receptor tyrosine kinase c-KIT [stem cell growth factor receptor (SCFR), CD117] expressed by melanocytes, mast cells, interstitial cells of Cajal, as well as hematopoietic stem cells and multipotent/common myeloid progenitors. Activating *KIT* mutations, KIT^{L576P} being the most common variant, are observed in 14 and 18 % of acral lentiginous and mucosal melanomas, respectively, but occurrence in skin melanomas is marginal (Tsao et al. 2012; Hodi et al. 2013; Kunz 2014). *KIT*-activation may also occur through gene amplifications observed in a significant percentage of melanoma cases. c-KIT dimerization with activation of its tyrosine kinase activity occurs upon extracellular binding of stem cell factor (SCF), a c-KIT ligand and cytokine involved in hematopoiesis, spermatogenesis, and melanogenesis. Melanoblast migration is a SCF/c-KIT controlled process through which SCF guides melanoblasts expressing c-KIT from the neural crest to their epidermal locations, and SCF also regulates survival

and proliferation of fully differentiated melanocytes. The activation of c-KIT leads to the activation of multiple signaling cascades, including the RAS/ERK, PI3-Kinase, Src kinase, and JAK/STAT pathways controlling cellular proliferation and survival in melanoma cells (Smalley et al. 2009). Moreover, c-KIT proliferative signaling depends on MITF activity in melanoma, where c-KIT stimulation leads to the activation of MITF specifically through the c-KIT phosphorylation sites Y721 (PI3 kinase binding site), Y568 and Y570 (Src binding site) (Phung et al. 2011).

Little is known about the role of c-Kit in redox modulation of melanomagenesis. SCF/c-KIT-dependent protection of keratinocytes from ROS-induced apoptosis has recently been demonstrated, and c-KIT anti-apoptotic activity regulating cellular redox state and loss of mitochondrial transmembrane potential have been described (Lee 1998; Lam et al. 2011). Interestingly, c-KIT redox mechanisms have been shown to play critical roles within stem cell niches, and c-KIT expression in cardiac precursor cells exhibits antioxidant activity through downregulation of NOX2 and its subunit p67(PHOX), while overexpression of NOX2 and NOX4 antagonizes *KIT* expression initiating cardiac lineage commitment and differentiation (Nadworny et al. 2013).

Due to the pronounced c-KIT-antagonism displayed by clinically used BCR-ABL-directed kinase inhibitor drugs, representing standard-of-care medications in chronic myelogenous leukemia, c-KIT-targeted pharmacological intervention for the treatment of acral lentiginous melanoma has quickly entered the stage of clinical practice (Hodi et al. 2013; Karimkhani et al. 2014).

13.4.5 AKT3-PTEN in Melanomagenesis and Redox Modulation

Activation of the PI3K pathway is a common genetic event in melanoma, with hyperactivation of the oncogenic kinase AKT3, observed in up to 60 % of sporadic melanomas, thought to originate from both *AKT3* (V-akt murine thymoma viral oncogene homolog 3; protein kinase B gamma) gene amplification and decreased activity of the AKT antagonist and tumor suppressor *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) (Tsao et al. 2012; Kwong and Davies 2013). Among three AKT isoforms (AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ) expressed in melanocytes and melanoma cells, AKT3 is the predominantly active member involved in cancer cell survival signaling, documented to be upregulated in 17 % of benign nevi, 43 % of dysplastic nevi, 49 % of primary melanomas, and 77 % of metastatic melanomas (Dai et al. 2005).

It has recently been shown that abrogation of $BRAF^{V600E}$-induced senescence by PI3K pathway activation contributes to melanomagenesis (Vredeveld et al. 2012). AKT3 has been shown to phosphorylate $BRAF^{V600E}$ attenuating the activity of the mutant protein to levels that promote rather than inhibit melanoma tumor progression, and acute shRNA-mediated depletion of PTEN prompted tumor progression in established murine $BRAF^{V600E}$-driven nevi. In the same study, pharmacologic

PI3K-inhibition in melanoma cells suppressed proliferation and induced the senescence-associated tumor suppressor $p15^{INK4B}$ eliminating subpopulations resistant to targeted $BRAF^{V600E}$ inhibition, findings that suggest feasibility of therapeutic reactivation of senescence and elimination of melanoma cells refractory to $BRAF^{V600E}$ by PI3K inhibition.

In support of a redox-directed involvement of AKT-signaling in melanomagenesis, it has been shown that the crucial conversion from radial growth phase (RGP) to the invasive vertical growth phase (VGP) of melanoma is orchestrated by AKT overexpression causing NAD(P)H oxidase 4 (NOX4)-dependent ROS production (Govindarajan et al. 2007; Liu-Smith et al. 2014). In malignant melanoma cell lines, NOX4 was instrumental in causing prooxidant deviations from redox homeostasis, and Nox4-directed siRNA intervention decreased ROS production with inhibition of anchorage-independent cell growth and tumorigenicity in nude mice. In melanoma patient samples, NOX4 expression was detectable in approximately one third of tumor specimens suggesting the association of NOX4 expression with melanomagenesis (Yamaura et al. 2009). It was also demonstrated that NOX4 expression supports the transformed phenotype by orchestrating the redox modulation of G_2/M cell cycle progression involving CDK1 and CDC25C. NOX4-dependent upregulation of constitutive oxidative stress and redox modulation of FAK (focal adhesion kinase) have been shown to control melanoma survival mediated via increased FAK-phosphorylation downstream of ROS-dependent inactivation of redox-sensitive phosphatases including PTEN and protein tyrosine phosphatases (Ribeiro-Pereira et al. 2014). More recently, a mechanism by which NOX4-dependent ROS production could be harnessed for the induction of TRAIL (TNF-related apoptosis-inducing ligand)-induced melanoma cell apoptosis was elucidated using the PI3-kinase inhibitor wortmannin causing a shift towards enhanced pro-apoptic (Thr167) and reduced anti-apoptotic phosphorylation (Ser184) of BAX (Quast et al. 2013). Targeting signaling through the PI3K-AKT pathway in malignant melanoma is an important goal of ongoing preclinical and clinical drug development efforts, and dual pathway inhibition using PI3K and MEK inhibitors that attenuate signaling through the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways is currently explored in ongoing clinical trials in malignant melanoma with *BRAF* or *NRAS* mutations (Britten 2013; Kwong and Davies 2013).

Experimental and clinical evidence suggests that expression and activity of the phosphatase PTEN is compromised in up to 43 % of melanoma patients (Mikhail et al. 2005). In the majority of melanomas *PTEN* impairment is caused by either chromosomal alteration, mutation, methylation-induced transcriptional silencing, or microRNA-dependent regulation (Tsao et al. 2012). In melanoma, PTEN expression has been shown to be a critical regulator of cell proliferation and survival acting through modulation of AKT3 phosphorylation status, and a murine spontaneous melanoma model ($BRAF^{V600E/+}$/$PTEN^{-/-}$/Tyr-CRE^{+}) combining expression of $BRAF^{V600E}$ with *PTEN* gene silencing elicits development of melanoma with 100 % penetrance, short latency, and metastases observed in lymph nodes and lungs (Dankort et al. 2009). Oxidation of the PTEN active site cysteine residue (C124) by ROS has long been recognized as a common mechanism regulating several key

phosphatases including PTEN, inactivating its lipid phosphatase and tumor suppressor functions as observed in multiple cancer cell lines (Kwon et al. 2004; Silva et al. 2008). However, redox modulation of PTEN has not been substantiated in melanoma cells.

13.4.6 RAC1 in Melanomagenesis and Redox Modulation

Recently, *RAC1*^{P29S} (Ras-related C3 botulinum toxin substrate 1) was identified as a recurrent UV-induced signature mutation in human melanoma tumors, representing the third most frequent gain-of-function mutation described in melanoma (Hodis et al. 2012; Tsao et al. 2012; Kunz 2014). Intriguingly, the mutated *RAC1*^{P29S} maintains intrinsic GTP hydrolysis and is spontaneously activated by substantially increased inherent GDP/GTP nucleotide exchange displaying high binding activity to downstream targets inducing melanocyte proliferation and migration (Davis et al. 2013). Rho GTPase family proteins (including RAC1, RhoA, Cdc42) are crucial regulators of cytoskeletal rearrangement and adhesion enabling cancer metastasis, and Rac1 is known to impact multiple cellular processes including actin-based cytoskeletal reorganization, DNA synthesis, cell transformation, motility, and -importantly- superoxide/ROS production thought to impact the aforementioned biological readouts. Overexpression of RAC1 has been observed in various tumour types, and expression of constitutively active RAC1 promotes cellular transformation, consistent with RAC1-dependent survival signaling through NFκB- and PI3K/AKT-dependent pathways (Singh et al. 2004). Pro-metastatic signaling via RAC-1 and ROS downstream of c-Met/hepatocyte growth factor receptor (HGF-R) proto-oncogene action has been demonstrated recently in melanoma, and catalytic SOD-mimetics (e.g. EUK-134) attenuated c-Met signaling-dependent ERK activation with inhibition of anchorage-independent growth, a finding consistent with a critical role of RAC1 and ROS in HGF/c-MET pro-metastatic signaling in melanoma (Ferraro et al. 2006). Importantly, RAC1 is a crucial activator of NOX1, and, intriguingly, NOX1 over-expression has been identified as a causative factor in ROS-dependent melanoma cell invasion characterized by induction of matrix metalloproteinase expression (MMP2, MMP9) and epithelial–mesenchymal transition (Liu et al. 2012; Liu-Smith et al. 2014). RAC1 activation upstream of NOX-dependent ROS formation has also been shown to control FAK (Focal Adhesion Kinase)-dependent motility in cultured melanoma cells (Kim et al. 2008). Based on the emerging mechanistic involvement of a RAC1-NOX1-ROS axis in melanoma invasiveness and the recent identification of activational oncogenic *RAC1* mutations in human melanoma tumors, it has been proposed that activation of RAC1 may represent a valuable biomarker of melanoma progression (Liu-Smith et al. 2014). Moreover, due to the availability of NOX-inhibitory therapeutics, RAC1- and NOX-dependent redox dysregulation represents an attractive melanoma drug target to be explored by future preclinical and clinical efforts (Block and Gorin 2012).

13.4.7 KEAP1 in Melanomagenesis and Redox Modulation

Recently, exome sequencing of acral lentiginous melanoma (ALM), a subtype harboring the *BRAF*^{V600E} mutation in only 10 % of cases, identified a somatic inactivating frameshift mutation in *KEAP1* (codon 507, exon 4), the gene encoding Kelch-like ECH-associated protein 1, a molecular change resulting in aberrant NRF2 (nuclear factor erythroid-derived 2-like 2) activation and increased intrinsic drug resistance characteristic of AML (Kunz 2014; Miura et al. 2014). KEAP1 is a redox-sensitive negative regulator of the basic leucine zipper (bZIP) transcription factor NRF2, a master regulator of the cellular antioxidant electrophilic stress response (Zhang 2010). KEAP1 is a substrate adaptor essential to Cul3-dependent ubiquitination of NRF2 followed by proteasomal degradation (Villeneuve et al. 2010). Oxidative or electrophilic stressors target critical cysteine residues in KEAP1, disrupting the KEAP1-Cul3 ubiquitination system leading to NRF2 accumulation followed by nuclear translocation and NRF2-dependent transcription of target genes with an ARE (antioxidant response element)-promotor sequence. Indeed, NRF2 target genes encode important regulators of cellular oxidative stress including components controlling biosynthesis and redox status of glutathione (e.g. *SLC7A11*, *GCLC*, *GCLM*, *GSR*), important determinants of cancer progression and chemoresistance (Zhang 2010; Mitsuishi et al. 2012a, b).

Strikingly, recent data have shown that constitutive activation of NRF2 can occur during later stages of tumorigenesis, representing a remarkable redox adaptation of cancer cells that enables chemoresistance and enhanced tumor cell survival under adverse conditions (Wang et al. 2008; Zhang 2010; Jaramillo and Zhang 2013). Moreover, the functional expansion of NRF2 may also contribute to metabolic reprogramming of cancer cells triggered by proliferative signals (Mitsuishi et al. 2012a, b). Consistent with these findings, the single nucleotide deletion observed in AML generates a mutant KEAP1 protein with impaired DGR/Kelch domain essential to KEAP1-NRF2 interaction, causing pronounced upregulation of NRF2 and resistance of AML cells to chemotherapeutics that impose electrophilic stress including cisplatin and dacarbazine. Due to the emerging role of NRF2 in redox adaptation of tumors, current drug discovery efforts aim at the development of NRF2 inhibitors for cancer cell chemosensitization (Ren et al. 2011).

13.4.8 MAP3K5 (ASK1) in Melanomagenesis and Redox Modulation

Recent data support the intriguing hypothesis that melanoma cells counterbalance elevated levels of oxidative stress that increase invasive and metastatic performance through down-regulation of oxidative stress-triggered cell death pathways including the ASK1 (apoptosis signal-regulating kinase 1; mitogen-activated protein kinase kinase kinase 5) pathway. Indeed, exome sequencing has identified frequent somatic

mutations in *MAP3K5* (and *MAP3K9*) in metastatic melanoma with 24 % of melanoma cell lines displaying mutations in the protein-coding regions of either *MAP3K5* or *MAP3K9* (Stark et al. 2012; Kunz 2014). Structural modeling of the kinase domain predicted the inactivating nature of the mutations, confirmed by in vitro biochemical assays that revealed reduced kinase activity of *MAP3K5*^{I780F} and *MAP3K9*^{W333*} variants. Vice versa, overexpression of *MAP3K5* or *MAP3K9* variants in HEK293T cells reduced phosphorylation of downstream MAP kinases. Furthermore, siRNA-based target attenuation of ASK1 in melanoma cells led to increased resistance to temozolomide treatment, suggesting that these mutations may cause chemoresistance in melanoma. ASK1 is a redox-sensitive member of the MAPKKK family, which activates both c-Jun N-terminal kinase (JNK) and p38 MAPK pathways and constitutes a pivotal signaling factor in oxidative stress-induced apoptosis (Kawarazaki et al. 2014). Reduced thioredoxin binds to ASK1 inhibiting its activity, and somatic mutations in *MAP3K5* have recently been shown to attenuate its pro-apoptotic function in melanoma through increased binding to thioredoxin (Prickett et al. 2014). Importantly, ASK1 may display dual functions as both tumor promoter and suppressor, opposing activities that depend on cell type and tissue context, and its specific role in melanomagenesis remains to be defined (Soga et al. 2012).

13.4.9 MYC in Melanomagenesis and Redox Dysregulation

The *MYC* proto-oncogene encoding the helix-loop-helix transcription factor c-MYC is involved in the control of proliferation, differentiation, and apoptosis, and structural alterations of the 8q24 chromosomal locus regulate *MYC* expression contributing to tumorigenesis. *MYC* activation is a frequent event in human melanoma representing a prognostic marker that can predict shorter overall survival (Chana et al. 1998, 2001; Grover et al. 1999). Indeed, upregulated chromosomal copy number changes at 8q24 harboring *MYC* are highly linked to poor prognosis in cutaneous malignant melanoma suggesting that *MYC* copy number gains play an important role in the aggressive clinical behavior of melanomas (Gerami et al. 2011; Pouryazdanparast et al. 2012). Moreover, c-MYC downregulation can sensitize melanoma cells to chemo- and radiotherapy, and high c-MYC expression enhances melanoma growth in SCID mice and facilitates interferon-α resistance in human melanoma (Biroccio et al. 2001; Tulley et al. 2004; Leikam et al. 2014; Meierjohann 2014).

It is now established that c-MYC overexpression plays an essential role in melanomagenesis through continuous suppression of oncogene (BRAF^{V600E} or NRAS^{Q61R})-induced senescence in melanoma cells enabling the crucial transition from benign to dysplastic nevus, and genetic attenuation of c-MYC expression has indeed been shown to induce senescence in metastatic melanoma cells (Michaloglou et al. 2005; Zhuang et al. 2008). c-MYC-driven evasion of oxidative stress-induced melanocyte senescence downstream of activated receptor tyrosine kinase signaling has been demonstrated, and a role of MIZ1 (Msx-interacting-zinc finger), another

zinc finger transcription factor and growth suppressing c-MYC interaction partner, in mediating melanocyte senescence was substantiated based on the observation that either c-MYC overexpression or MIZ1 knockdown antagonized senescence with attenuation of endogenous ROS formation and DNA damage (Leikam et al. 2014; Meierjohann 2014). As a mediator of the oxidative stress-directed effects of c-MYC and MIZ1 modulation, the c-MYC target gene *CTH* (encoding cystathionase), an enzyme involved in the control of cellular L-cysteine and glutathione synthesis, was identified. Consistent with a causative role of *CTH* as new c-MYC target gene with an important function in senescence evasion, pharmacological or genetic *CTH* target inhibition reestablished senescence in human melanoma cells attenuating melanoma cell proliferation, H_2O_2 resistance, and soft agar growth.

Multiple lines of experimental evidence indicate that c-MYC counteracts oxidative stress through various molecular effectors. For example, the transcription factor and master regulator of the cellular antioxidant response Nrf2 is induced by c-MYC, and previous research has shown that c-MYC controls cellular glutathione levels in melanoma cells (DeNicola et al. 2011; Leikam et al. 2014). Moreover, *FOXM1*, a target gene of c-MYC encoding a member of the FOX family of transcription factors, stimulates the expression of superoxide dismutase, catalase and peroxiredoxins and thus contributes to antioxidant senescence evasion (Li et al. 2008).

Cumulative evidence suggests that c-Myc regulates the intracellular iron pool, and c-MYC-regulation of iron-controlling genes is essential for cell proliferation and transformation (Wu et al. 1999; O'Donnell et al. 2006). Overexpression of the c-MYC target gene *TFRC1* encoding the transferrin receptor TFR1, a key mediator of cellular iron uptake causatively involved in hyperproliferation and tumorigenesis is a common feature of human malignancies (O'Donnell et al. 2006). c-MYC-driven tumorigenesis is generally associated with *TFRC1*-upregulation, and in melanoma tissue, TFR1 is highly upregulated in primary and metastatic tumors but not detectable in benign melanocytic nevi (Soyer et al. 1987; van Muijen et al. 1990; Ostmeier et al. 2001). Recent research has demonstrated that in melanoma cells *TFRC1* expression is not responsive to pharmacological (vemurafenib) or genetic (siRNA) modulation targeting ^{V600E}BRAF (Packer et al. 2009), a finding consistent with a c-MYC-driven mechanism of intracellular iron regulation as observed in other tumor types such as Burkitt's lymphoma (Habel and Jung 2006). Histochemical analysis has detected increased iron levels in malignant melanoma tissue compared to benign nevi (Bedrick et al. 1991), potentially originating from c-Myc driven dysregulation of iron homeostasis.

13.4.10 Oncogene-Driven Iron Dysregulation: An Opportunity for Synthetic Lethal Refinement of Redox Intervention Targeting Malignant Melanoma

Molecularly targeted intervention is currently revolutionizing cancer chemotherapy, promising unprecedented therapeutic benefit based on superior selectivity and width of therapeutic window (Hoeflich et al. 2006; Tsai et al. 2008; Yang et al. 2010;

Chapman et al. 2011). Among emerging molecular strategies that enable targeted intervention, the concept of 'synthetic lethality' has attracted considerable attention (Tong et al. 2001; Chan and Giaccia 2011). Originating from genome-wide studies that demonstrated the existence of synthetic-lethal interactions in yeast deletion strains, where two genes are considered synthetic lethal if perturbations in either alone has little consequence, but simultaneous perturbation of both causes cell death, the concept of 'synthetic lethality' has recently enabled strategies for the identification of molecularly targeted cancer therapeutics (Chan and Giaccia 2011).

Synthetic-lethal drug screening has identified chemotherapeutics that cause cytotoxicity confined to cancer cells with loss of function mutations in tumor suppressor genes or upregulated oncogene expression (Ferrari et al. 2010). For example, a synthetic-lethal mechanism of action determines selectivity of PARP-1 inhibitors targeting *BRCA1* loss of function mutants in breast carcinoma, but no synthetic-lethal molecular strategy is currently available for melanoma chemotherapy. As another example of a recently identified synthetic-lethal relationship with particular relevance to melanoma, the p110 Cut homeobox 1 (CUX1) transcription factor *CUX1* was found in a genome-wide RNAi screen defining synthetic lethal interactions with oncogenic *RAS* (Ramdzan et al. 2014). *CUX1* function in base excision repair as an ancillary factor for the 8-oxoG-DNA glycosylase *OGG1* is consistent with the finding that *RAS* transformation requires *CUX1*-dependent repair of oxidative DNA damage since elevated ROS levels in cells with sustained *RAS* pathway activation can cause cellular senescence, a molecular vulnerability of *RAS*-transformed cells that may be exploited by future drug discovery efforts.

Despite the causative role of c-MYC dysregulation in melanomagenesis (Chana et al. 2001; Zhuang et al. 2008; Gerami et al. 2011; Pouryazdanparast et al. 2012), the concept of synthetic-lethal intervention has not yet been harnessed for pharmacological strategies that aim at apoptotic eradication of c-MYC overexpressing melanoma cells. Recently, unbiased synthetic-lethal screening has identified novel molecular therapeutics that target oncogene-expressing cancer cells through induction of iron-dependent cytotoxic oxidative stress without causing toxicity in untransformed cells (Yang and Stockwell 2008). Altered iron homeostasis in cancer cells may originate from either oncogenic activation (e.g. *RAS*, *MYC*) or tumor suppressor gene loss of function (e.g. *TP53*); these genetic alterations upregulate the intracellular labile iron pool (LIP) through altered expression of transferrin receptor 1 (TFR1), iron regulatory proteins (IRPs), and ferritin. Importantly, increased availability of redox-active labile iron sensitizes these cells to specific redox-directed drugs. This 'synthetic-lethal' vulnerability is confined to malignant cells that display transformation-associated alterations increasing the intracellular redox-active LIP (Yang and Stockwell 2008; Wondrak 2009). The sesquiterpene endoperoxide artemisinin and other semisynthetic artemisinin-derivatives constitute an important class of FDA-approved antimalarial drugs that kill plasmodium parasites through induction of iron-dependent oxidative stress (Chaturvedi et al. 2010). Artemisinin-based cytotoxic activities targeting cancer cells in vitro and in vivo are well documented, attributed to iron-dependent activation of the endperoxide-pharmacophore that causes cell death downstream of free radical and reactive oxygen species formation (Wondrak 2009; Chaturvedi et al. 2010). In search for novel experimental

therapeutics that might eliminate melanoma cells but not melanocytes through targeted induction of oxidative stress, we have recently performed pilot studies documenting melanoma cell-selective apoptogenicity of artemisinin-antimalarials that was blocked by antioxidant, iron-chelating, or si*TFR1*-based intervention supporting the hypothesis that a synthetic-lethal relationship exists between oncogene-driven iron dysregulation and artemisinin-sensitivity of malignant melanoma cells (Wondrak 2009; Cabello et al. 2012). Additional preclinical data suggest feasibility of repurposing clinically used artemisinins and other redox-directed antimalarials including amodiaquine for pro-oxidant and autophagy-directed antimelanoma intervention (Qiao et al. 2013).

13.5 Synopsis

An urgent need exists for improved chemotherapeutic options targeting the metastatic stage of malignant melanoma that remains incurable by currently available treatments. The list of gene alterations that play a causative role in melanomagenesis is rapidly expanding, and cumulative evidence suggests an important mechanistic role of melanomagenic genomic changes in the control of cellular redox homeostasis and signaling, relevant to suppression or promotion of tumorigenesis. It is now evident that redox-directed functions of specific melanomagenic genes including *BRAF* and *MYC* may create synthetic-lethal dependencies and molecular vulnerabilities that can be targeted by redox- or metabolism-directed therapeutics even if resistance to kinase-directed therapeutics has occurred; it remains to be seen if redox therapeutics can benefit melanoma patients.

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Chapter 14
Targeting Hypoxic Adaptations of Cancer Cells: Molecular Mechanisms and Therapeutic Opportunities

Ceen-Ming Tang and Jun Yu

Abstract Hypoxia is a common feature in tumours. In the majority, it confers an adverse prognosis owing to its contributions to angiogenesis, growth, invasion, metastasis, and chemoresistance. Given its central role in tumorigenesis, tumour hypoxia is an attractive therapeutic target. There are three main O_2-sensing pathways, namely the unfolded protein response (UPR), the target of rapamycin kinase (mTOR), and the hypoxia-inducible factor-1 (HIF-1) pathway. Overwhelming evidence suggests that hypoxic adaptations in tumour cells converge onto the hypoxia-inducible factor-1 (HIF-1) pathway. Overexpression of HIF-1α is thus associated with resistance to cancer chemotherapy and increased patient mortality in several cancer phenotypes. In the present chapter, we summarize the role of intratumoral hypoxia and bioactive lipids in enhancing HIF-1 activity, critically discussing the potential for HIF-1α inhibitors in cancer chemotherapy. Additionally, we consider the therapeutic value of HIF-independent targets such as the UPR and mTOR signalling pathways, and discuss the use of new drug delivery systems. Considering pre-clinical studies, HIF-1 inhibitors appear to have anti-tumour effects and thus represent a novel therapeutic strategy.

Keywords Hypoxia • Normoxia • Tumour microenvironment • Bioactive lipids • Hypoxia-inducible factor-1 • Unfolded protein response • mTOR • von Hippel-Lindau

C.-M. Tang
Department of Pharmacology, University of Oxford, Oxford, UK

Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, Institute of Digestive Disease, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, Hong Kong

J. Yu (✉)
Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, Institute of Digestive Disease, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong, Hong Kong

Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong
e-mail: junyu@cuhk.edu.hk

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protein • Metabolic reprogramming • HIF-1 inhibitors • Targeted drug delivery • Combination therapy • Patient selection

14.1 The Hypoxic Tumour Microenvironment

Many human cancers contain regions of hypoxia due to rapid cell proliferation and the presence of intratumoral blood vessels that are structurally and functionally abnormal. Notably, the presence of intratumoral hypoxia is associated with an increased risk for invasion, metastasis, treatment failure, and patient mortality (Semenza 2007). Tumour cells have multiple elaborate, evolutionarily conserved mechanisms that enables them to respond to hypoxia in the tumour microenvironment. There are three main O_2-sensing pathways, namely the unfolded protein response, the target of rapamycin kinase (mTOR), and the hypoxia-inducible factor-1 (HIF-1) pathway. Together, these three pathways influence the phenotype of hypoxic cells by altering angiogenesis, cellular growth and autophagy, as well as endoplasmic reticulum (ER) homeostasis to promote hypoxia tolerance.

Overexpression of HIF-1α is associated with an aggressive phenotype and increased mortality in many cancer types (Zhong et al. 1999). Numerous transforming viruses linked to tumorigenesis increase HIF-1 expression – examples include the hepatitis B virus (HBV) X protein (Yoo et al. 2004), human papillomavirus (HPV) E6/E7 oncoproteins (Nakamura et al. 2009), and latent membrane protein 1 (LMP-1) from Epstein-Barr virus (EBV) (Kondo et al. 2006). By increasing HIF-1α synthesis (Wakisaka et al. 2004) and degradation of the prolyl hydroxylases (Kondo et al. 2006), LMP-1 promotes tumorigenicity through inducing a HIF-1 dependent de-differentiated phenotype characteristic of cancer progenitor cells (Helczynska et al. 2003; Kondo et al. 2011). LMP-1 also up-regulates glycolytic enzymes – rapidly proliferating cancer cells depend on the Warburg effect to generate sufficient glycolytic intermediates for anabolic metabolism (Darekar et al. 2012). LMP-1 mediated up-regulation of matrix metalloproteinase 9 (MMP-9), which digests the basement membrane, further facilitates cellular invasion and metastasis (Wakisaka and Pagano 2003). Consequently, oncoviruses may cause HIF-1 overexpression and potentiate tumorigenesis.

In addition to hypoxia and infection by oncoviruses such as EBV, HBV, and HPV, HIF-1 expression may also be up-regulated by bioactive lipid mediators. The role of eicosanoids in carcinogenesis was first elucidated in epidemiological studies whereby a reduced risk for colon cancer was observed in long-term users of non-steroidal anti-inflammatory drugs (NSAIDs). Derived from the enzymatic action of cyclooxygenase (COX) on arachidonic acid, levels of prostaglandin E2 (PGE_2) were shown to be elevated in immunohistochemical analyses of gastric and colon cancer surgical biopsies. Subsequent exposure of human HCT116 colon and PC-3ML prostate cancer cells to PGE_2 revealed that PGE_2 induced vascular endothelial growth factor (VEGF) expression in a HIF-1α dependent process. In a potential positive feedback loop, activation of the PGE_2 receptor EP1 in HepG2 hepatocellular carcinoma cells up-

regulates normoxic expression of HIF-1α, which then binds to the HRE within the COX-2 promoter to further enhance PGE_2 production and promote tumourigenesis. In support of this hypothesis, increased cell proliferation and tube formation of human umbilical vein endothelial cells (HUVECs) was observed in an AGS gastric cancer cell line transfected with a COX-2 expression vector. Critically, the consequent HIF-1α protein accumulation and subsequent angiogenic effects on HUVECs were reduced by NS-398 (COX-2 inhibitor), SC19220 (PGE_2 receptor antagonist), and antisense HIF-1α transfection. Thus the COX-2/PGE_2/HIF-1α/VEGF pathway may be critical in angiogenesis and tumour progression. Other eicosanoids implicated in HIF-1 up-regulation include leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETEs) generated by lipoxygenases (LOX). Specifically, PC-3 human prostate cancer cells with 12-LOX overexpression exhibited elevated nuclear HIF-1α levels, with the consequent increase in expression of VEGF and the glucose transporter GLUT-1 further enabling survival of tumour cells under hypoxic conditions. Enhanced HIF-1α accumulation under normoxic conditions may alternatively be attributed to reactive oxygen species (ROS) dependent stabilization of HIF-1α by sphingosine-1-phosphate (S1P), an oncogenic bioactive lipid mediator which modulates angiogenesis, cell proliferation, and apoptosis. Indeed, overexpression of sphingosine-1-kinase (SphK1) was observed in prostate PC-3, brain U87, and lung A549 cancer cells, and is correlated with poor prognosis in patients with glioblastoma. Moreover, inhibition of SphK1 with siRNA or pharmacological antagonists prevented the accumulation of HIF-1α and its downstream transcriptional activity. Thus bioactive lipid mediators play an important role in HIF-1 overexpression and tumour progression (Tang and Yu 2013).

HIF-1 may also be over-expressed due to genetic mutations, such as gain-of-function mutations in oncogenes (AKT), or loss of function for tumour suppressor genes (PTEN, VHL). For example, HIF-1α is frequently overexpressed in prostate cancer due to loss of PTEN, which leads to hyper-activation of the AKT/mTOR pathway, which promotes HIF-1 signalling. Receptor tyrosine kinase-dependent signalling pathways, including EGFR, HER2/Neu, PI3K/AKT/mTOR, and MAPK pathways have also been implicated in the induction of HIF-1α. As HIF-1 mediates multiple tumour survival mechanisms and its overexpression arises not only because of the actions of bioactive lipid mediators, viral infections, and intratumoral hypoxia but also through oncogene gain-of-function and tumor suppressor gene loss-of-function mutations, HIF-1 may represent a final common pathway in cancer pathogenesis. Hence, HIF-1 is an attractive target for cancer therapy.

14.2 Molecular Biology of Hypoxia-Inducible Factor

The transcription factor HIF-1 is a heterodimeric protein composed of an O_2-regulated HIF-1α and a constitutively expressed HIF-1β subunit. Under normoxic conditions, HIF-1α is hydroxylated on proline residue 402 and/or 564, which is required for binding of the von Hippel-Lindau (VHL) protein, the recognition

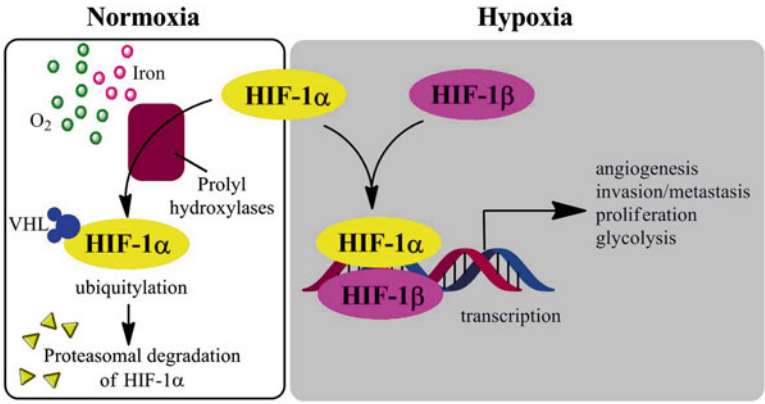

Fig. 14.1 Mechanisms of hypoxia-inducible factor (HIF)-1α regulation under aerobic and hypoxic conditions. *VHL* von Hippel-Lindau

subunit of an E3 ubiquitin ligase that targets HIF-1α for proteasomal degradation (Fig. 14.1). An additional control over HIF activity is provided by a factor inhibiting HIF-1 (FIH-1), which hydroxylates asparagine residue 803 to inhibit the association between HIF-1α and CBP/p300 co-activators to inhibit its transcriptional activity. Critically, these hydroxylation reactions require α-ketoglutarate and O_2 as substrates. Enzyme activity is thus limited by hypoxic conditions, enabling HIF-1α to accumulate and dimerize with HIF-1β. The functional transcription factor then translocates to the nucleus, where it binds at the core hypoxia response element 5′-RCGTG-3′ to induce genes involved in angiogenesis, glycolysis, de-differentiation, invasion, and metastasis (Tang and Yu 2013).

14.3 The Role of HIF-1 in Tumorigenesis

Data from genome-wide chromatin immunoprecipitation coupled to next-generation high-throughput screening (ChIP-seq) studies suggest that HIF directly regulates over 800 genes involved in many critical aspects of cancer biology (Schödel et al. 2011). In response to hypoxia, HIF-1 induces expression of growth factors such as insulin-like growth factor-2 (IGF-2) and transforming growth factor-α (TGF-α). These growth factors bind to their cognate receptors to promote cell proliferation/survival, as well as the expression of HIF-1α itself through autocrine-signalling pathways.

This growth is supported by HIF-1 dependent expression of erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which facilitates erythropoiesis and angiogenesis to increase the oxygen carrying capacity and vascular density respectively. Numerous HIF-1 dependent metabolic reprogramming mechanisms also occur. Experimental evidence from HIF-1α knockout mice unequivocally demonstrated that HIF-1 up-regulates expression of all glucose transporters (GLUT1, GLUT2), glycolytic enzymes (GAPDH, LDHA), and glycogen

storage enzymes (GYS1, GBE1). It also orchestrates a subunit switch in cytochrome c oxidase to increase the efficiency of the electron transport chain, enabling continued respiration in conditions of moderate hypoxia without increased levels of reactive oxygen species (ROS). HIF-1 further up-regulates the expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates pyruvate dehydrogenase (PDH) under severe hypoxia to inhibit oxidation of pyruvate into acetyl-CoA for entry into the TCA cycle. To compensate for the reduced flux of glucose to citrate, reductive carboxylation of glutamine is used to generate cytosolic citrate for fatty acid synthesis. Collectively, these changes facilitate the switch from mitochondrial oxidative metabolism to anerobic glycolysis to maintain an undifferentiated state under hypoxic conditions (Semenza 2013).

HIF-1 also promotes epithelial-mesenchymal transition by inducing transcription of gene coding repressors (ID2, ZEB2) which mediates the loss of E-cadherin and other proteins that contribute to maintaining the cellular cytoskeleton and cell-cell adhesion. It also induces transcription of LOX and matrix metalloproteinases (MMPs), which augments hypoxia-induced invasion of tumour cells and formation of metastatic lesions in animal models.

14.4 HIF-1 as a Therapeutic Target in Cancer

HIF-1 over-expression in human cancer is associated with poor survival. Immunohistochemical analyses of oropharyngeal (Aebersold et al. 2001) and esophageal squamous-cell carcinoma (Tzao et al. 2008), as well as laryngeal (Schrijvers et al. 2008), gastric (Griffiths et al. 2007; Takahashi et al. 2003), pancreatic (Sun et al. 2007), colorectal (Rajaganeshan et al. 2008), and rectal carcinoma (Rasheed et al. 2009) biopsies also revealed a positive correlation between HIF-1 overexpression, radiotherapy resistance, and increased patient mortality.

The importance of HIF-1 in tumour growth is emphasized by experimental data in which overexpression of HIF-1α in HCT116 colorectal cancer (Ravi et al. 2000) and PCI-10 pancreatic cancer cells (Akakura et al. 2001) increased vessel density and tumour growth respectively. Critically, previous studies have demonstrated that HIF-1α null mutations severely impede tumour growth by reducing expression of glycolytic enzymes, with tumours unable to grow beyond 2 mm^3 unless supported by neovascularization mediated by hypoxic induction of HIF-1 (Ma and Adjei 2009; Ryan et al. 1998). In contrast to VEGF inhibitors, HIF-1 inhibitors were also shown to decrease breast cancer cell metastasis in mouse orthotopic transplantation models (Zhang et al. 2012), and sensitize tumours to radiotherapy (Moeller et al. 2004). Additionally, anti-sense blockade of HIF-1α expression in gastric carcinoma cells reduced VEGF production in response to COX-2 overexpression (Huang et al. 2005), with the introduction of HIF-1α siRNA into a glioma cell line down-regulating MMP-2/MMP-9 to suppress cell migration and invasion into adjacent normal tissue (Fujiwara et al. 2007). The siRNA also selectively prevented hypoxia-induced treatment resistance (Sullivan et al. 2008).

Since HIF-1 mediates multiple tumour survival mechanisms, and its overexpression arises not only due to the actions of bioactive lipid mediators, viral infections, and intratumoral hypoxia, but also through oncogene gain-of-function (Giatromanolaki et al. 2004) and tumour suppressor gene (Maxwell et al. 1999) loss-of-function mutations, HIF-1 may represent a final common pathway in cancer pathogenesis. Therefore, HIF-1 is an attractive target for cancer therapy.

14.4.1 Pharmacological Targets in the HIF-1 Pathway

Small-molecule inhibitors of HIF-1 are highly desirable due to its central role in tumorigenesis. Broadly, small-molecules may inhibit HIF-1 by decreasing its protein levels, or by preventing its dimerization, DNA-binding, or transactivation (Fig. 14.2).

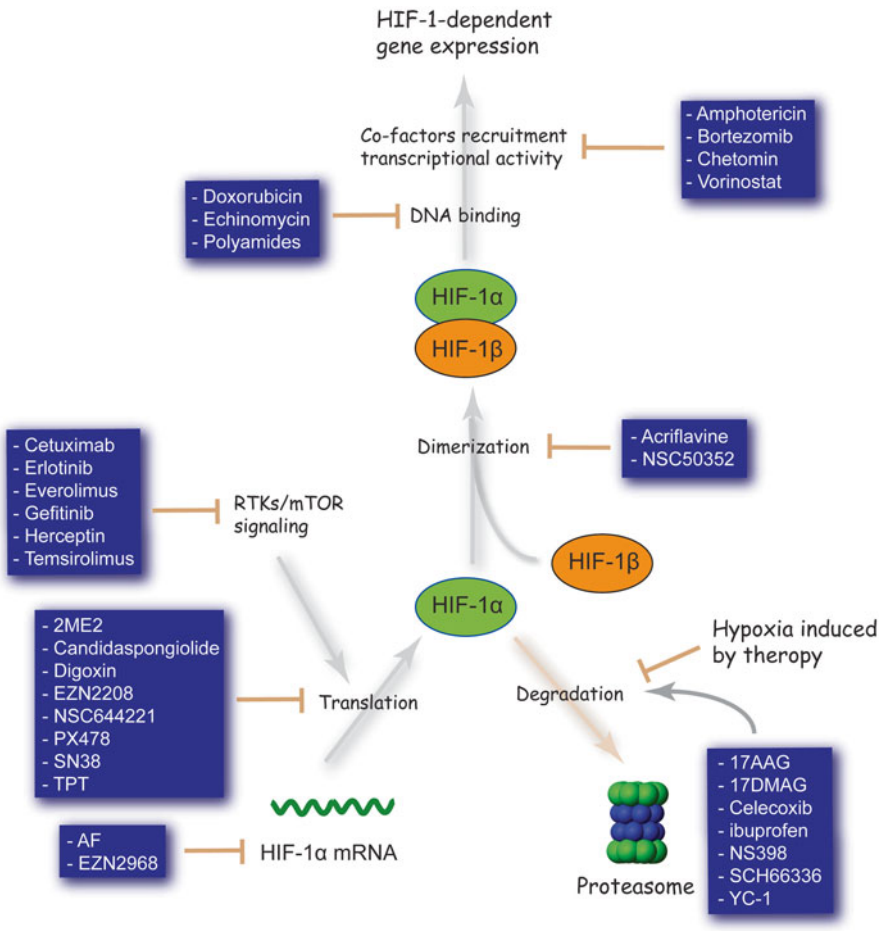

Fig. 14.2 HIF-1 inhibitors. These are meant to be illustrative, rather than comprehensive, examples

Sorafenib, and P3155 have been found to decrease HIF-1 synthesis (Liu et al. 2012; Manohar et al. 2011), with systemic digoxin treatment in tumour-bearing mice blocking lymphatic metastasis to axillary lymph nodes (Schito et al. 2012). Other examples include KC7F2 and PX-478, which inhibits translation via a VHL- and p53-independent mechanism (Koh et al. 2008; Narita et al. 2009), and the RNA antagonist EZN-2968, which inhibits expression of HIF-1α mRNA (Greenberger et al. 2008). Notably, PX-478 and EZN-2968 cause dose-dependent reductions in levels of HIF-1α, VEGF secretion, and tumour size in xenograft models, and were both well tolerated in Phase I clinical trials (Greenberger et al. 2008; Jeong et al. 2014; Koh et al. 2008). Further modulators identified to date include geldanamycin, which reduces HSP90 binding to HIF-1α to destabilize folding and increase proteasomal degradation (Isaacs et al. 2002), and the mTOR inhibitor rapamycin. Indirect actions through upstream or downstream signaling pathways however cause many side effects. The development of novel specific HIF-1 inhibitors is thus needed.

To identify HIF-1 inhibitors with greater selectivity, there are on-going efforts to target the protein-protein interactions. Traditionally considered to be an undruggable target due to its weak and transient nature, preventing HIF-1 heterodimerization is a challenging but potentially rewarding strategy. The small-molecule acriflavine, which inhibits HIF-1α and HIF-1β dimerization, acts as a radiosensitizer and potently inhibited prostate cancer xenograft growth and breast cancer metastasis to the lungs in mouse models (Lee et al. 2009b; Lim et al. 2012). The new peptide cyclo-CLLFVY may hold more promise in this regard. Using a high-throughput genetically encoded screening platform, cyclo-CLLFVY was found to bind to the PAS-B domain of HIF-1α. It mediated selective inhibition of the HIF-1 mediated cellular response by disrupting dimerization in MCF-7 and U2OS cell lines (Miranda et al. 2013). However, cyclic peptides have difficult pharmacokinetics, making the transition to clinical use challenging.

In an alternate approach, echinomycin and anthracycline antibiotics may be used to inhibit HIF-1: DNA-binding via DNA intercalation at the hypoxia response element (HRE) (Kong et al. 2005; Lee et al. 2009a). DNA intercalators however show limited sequence specificity, thus causing off-target effects. Indeed, echinomycin cannot be used in cancer chemotherapy due to its secondary action on Sp1 (5′-CCGCCC-3′), which ultimately increases HIF-1α expression under normoxic conditions. A more promising strategy may be to target the DNA-binding domain of HIF-1α (unpublished work).

14.4.2 Limitations of Previous Approaches

Numerous cancer chemotherapeutic agents target angiogenesis in tumours to reduce primary tumour growth. Response to anti-VEGF therapy has however been poor, as intratumoral hypoxia arising from impaired angiogenesis causes HIF-1 dependent metastasis and expansion of cancer stem cell pools (Conley et al. 2012). Hence targeting HIF-1 activity may abrogate the compensatory pathways required for cancer cell survival.

Indeed, it has been shown that bevacizumab increases intratumoral hypoxia and expression of HIF-dependent genes in glioma xenografts, and that inhibition of HIF-1α by topotecan significantly reduces tumour cell proliferation (Rapisarda et al. 2009). Critics, however, argue that topotecan has failed to show any anti-cancer effects in previous clinical trials. The key distinction is that HIF inhibitors were previously used as cytotoxic agents and administered episodically at the maximum tolerated dose, whereas continuous inhibition of HIF activity requires frequent administration at lower doses. This is supported by evidence from a recently completed pilot study involving 16 patients with advanced cancer and biopsy-proven HIF-1α overexpression, where topotecan resulted in decreased tumour blood flow in seven out of ten patients as measured by dynamic contrast enhanced magnetic resonance imaging (DCE-MRI), and a loss of HIF-1α expression in four out of seven patients (Kummar et al. 2011). Further research is needed to determine the optimal dosing schedule for chronic use of HIF inhibitors as anti-cancer agents.

14.4.3 Single Agent Versus Combination Therapy

There is overwhelming evidence for the use of HIF-1 inhibitors in combination therapy. Hypoxia promotes selection of a treatment resistant phenotype by promoting transcription of genes involved in multidrug resistance (MDR-1, ABC transporters), and down-regulating genes involved in DNA mismatch repair. However, tumour cells have heterogeneous expression of HIF-1, thus conceptually, HIF-1 inhibitors used as a single agent may be ineffective. In support of this hypothesis, experimental evidence from combination treatment with rapamycin and LBH589, a mTOR and HDAC inhibitor respectively, had significantly greater anti-angiogenic and anti-tumour activity in PC3 and C2 cell lines in vivo compared with single agents (Verheul et al. 2008). Similarly, addition of low dose daily topotecan – a HIF inhibitor – to bevacizumab significantly inhibited tumour growth and reduced microvessel-density relative to mice treated with topotecan or bevacizumab alone in U251-HRE xenografts ($p<0.01$) (Rapisarda et al. 2009). It is plausible that the increase in cytotoxic activity of combination therapy is due to inhibition at different levels of HIF-1α regulation, or by inhibition of other oncogenic pathways.

The role of HIF inhibitors on the effects of radiotherapy for hypoxic tumours is less clear. HIF inhibitors given prior to treatment may suppress the effects of radiotherapy because its anti-angiogenic effects increases the radio-resistant hypoxic fraction. However, pre-clinical studies have also demonstrated an inverse correlation between hypoxia and local tumour control after irradiation. Examples include the use of HIF inhibitors BAY-84-7296 and YC-1 in combination with radiotherapy, both of which significantly enhanced the effects of radiotherapy against tumour xenografts if given after the radiation had been delivered. This may be due to hypoxia-induced expression of pro-angiogenic factors, which promote survival of endothelial cells following radiation, or through protection of cancer stem cells. Hence the timing of the treatment regime regarding the use of HIF inhibitors in combination with radiotherapy is critical (Harada et al. 2009).

14.4.4 Contraindications to Treatment with HIF-1 Inhibitors

One potential caveat is that patients with anemia or severe ischemic cardiovascular disease may experience exacerbation of their condition during treatment. In support of this hypothesis, post-hoc analysis from the PROactive randomized controlled trial showed the pioglitazone – an inhibitor of autocrine and paracrine angiogenesis through mitochondrial stabilization of HIF-1 – was associated with critical limb ischemia and an increased hazard for surgical or percutaneous lower extremity revascularization in diabetic patients (Dormandy et al. 2009). However, this is only a relative contraindication for its use.

14.5 Exploring HIF-Independent Strategies

Although the most studied cellular response to hypoxia is mediated by the transcription factor HIF-1, several HIF-independent mechanisms have also been implicated in hypoxic adaptation. These include the unfolded protein response (UPR) and the mTOR signalling pathway, which curtail oxygen consumption by energy expensive processes such as protein synthesis. Given their pivotal role in cellular adaptation to hypoxic stress, they are of considerable interest as therapeutic targets.

14.5.1 The Unfolded Protein Response (UPR)

Hypoxia, through the lack of oxygen to act as the terminal electron acceptor in the redox relay for disulphide bond formation, impairs protein folding in the ER. Accumulation of unfolded proteins within the ER activates the UPR, which mediates a global reduction in transcription and translation through the PERK-eIF2A-ATF4, IRE1-XBP1, and ATF6 signalling pathways. Clinical data suggests that all three branches of the UPR are up-regulated in human cancers. ATF4 over-expression has been found in brain, breast, cervical, and skin cancers as compared to adjacent normal controls (Bi et al. 2005). Levels of spliced XBP1 and ATF6 mRNA were also elevated in hepatocellular carcinoma samples (Shuda et al. 2003). Critically, transformed mouse embryo fibroblasts (MEFs) derived from PERK- and XBP1- knockout mice exhibit reduced clonogenic survival after hypoxic stress, as well as impaired tumour growth (Bi et al. 2005; Romero-Ramirez et al. 2004). Similar results were observed following transfection of short hairpin RNA (shRNA) against ATF4 into HT1080 human fibrosarcoma cells (Ye et al. 2010). These studies support the concept of targeting the UPR to inhibit tumour growth.

Two therapeutic strategies are currently being pursued. One approach is to inhibit the UPR by targeting its components PERK, ATF4, and IRE1. Salicylaldehyde analogues and the small-molecule GSK2606414A have recently been identified as potent and selective inhibitors of IRE1 and PERK respectively (Axten et al. 2012;

Volkmann et al. 2011). The antidiabetic biguanides, such as metformin, also inhibit production of UPR transcription activators XBP1 and ATF4 to induce cell death under conditions of glucose deprivation (Saito et al. 2009). Significantly, metformin re-profiled to treat cancer would have an immediate clinical impact. A second approach seeks to exacerbate ER stress in order to overwhelm the UPR, shifting the balance in favour of pro-apoptotic functions. In support of this therapeutic strategy, ER stressors such as thapsigargin or the clinically approved proteasome inhibitor bortezomib exhibit hypoxia-selective cytotoxicity (Fels et al. 2008). Chloroquine, which inhibits autophagy to aggravate ER stress, has also been shown to reduce the fraction of viable hypoxic tumour cells and increase tumour response to radiation (Rouschop et al. 2010). In summary, the UPR is an important mediator of the hypoxic microenvironment, and therapeutic strategies to inhibit the UPR shows promising anti-tumour effects.

14.5.2 The mTOR Signalling Pathway

mTOR senses cellular energy, nutrient, and oxygen levels to regulate cell growth and survival. Under hypoxic conditions, mTOR complex 1 (mTORC1) kinase activity is inhibited by activation of the tuberous sclerosis protein I (TSC1)-TSC2 complex through activation of AMP-activated protein kinase (AMPK) or transcriptional up-regulation of regulated in development and DNA damage responses 1 (REDD1). Similarly, the hypoxia-inducible pro-apoptotic protein BNIP3 is reported to inhibit mTORC1 by direct binding to RAS homologue enriched in brain (RHEB). The resulting suppression of mTORC1 causes hypophosphorylation of 4E-BP1, which leads to its increased association with the cap-binding protein eIF-4E and inhibition of cap-dependent translation. The extent of this inhibition depends on the duration and severity of hypoxia.

It is hypothesized that hypoxia has a dual-role in tumorigenesis. In small, early stage tumours, moderate hypoxia (~1 % O_2) should suppress tumour growth through negative regulation of mTORC1. Accumulating evidence, however, suggests that the mTOR signalling network is frequently dysregulated in human cancers. This is corroborated by experiments in two independent p53 null fibroblast lines, whereby the resulting decrease in REDD1 expression reduced sensitivity to oxidative stress (Ellisen et al. 2002). Thus, it is plausible that hypoxia-driven inhibitory mechanisms for down-regulating the mTOR pathway are impaired in tumour cells (Schneider et al. 2008).

Several studies have investigated the activity of pharmacological mTOR inhibitors in hypoxic cells. Rapamycin and its derivatives (rapalogues) crosslink with the immunophilin FK506-binding protein (FKBP12) to form a complex which binds the FKBP-rapamycin binding domain of mTOR to inhibit mTORC1 allosterically. It further inhibits HIF-1α accumulation to mediate hypoxia-selective anti-proliferative effects. The success of rapalogues in clinical trials have however been limited. This may be due to stimulation of the anti-apoptotic PI3K-AKT pathway,

as a result of mTORC1 inhibition and up-regulation of IGF-1R signalling. In support of this hypothesis, tissue samples from patients with colon or breast cancer after 4 weeks of treatment with rapalogues had higher levels of activated AKT compared to pre-treatment samples (O'Reilly et al. 2006). Significantly, rapalogue treatment also lead to MAPK activation through a PI3K-dependent feedback loop (Carracedo et al. 2008). Hence dual mTOR/PI3K inhibitors may be a more effective therapeutic approach.

mTORC1 suppression by severe hypoxia (~0.1 % O_2) in late stage tumours, however, may be an adaptive response in face of energy limitations which paradoxically favours hypoxic cell survival. The consequences of mTOR inhibition are thus difficult to predict without appropriate patient stratification. More alarmingly, there is significantly interplay between the mTOR, UPR, and HIF-1 signalling pathways. Given its central role in other aspects of cell growth and metabolism, mTOR presents as a less well-defined opportunity to target hypoxic cell survival.

14.6 Perspectives

Therapeutic opportunities in targeting tumour hypoxia have yet to be fully realized. This is despite identification of numerous molecular targets, including HIF-1, the UPR, and the mTOR signalling pathway. From a drug discovery point of view, a better understanding of the interplay between these molecular targets are needed to design drugs with greater selectivity. Improvements in drug delivery systems are also needed to facilitate selective tumour targeting. The high rates of attrition in the pipeline, however, remains the most important issue to be addressed. Thus drug development in the future should consider using re-profiling techniques to reduce failure due to toxicity. Moreover, clinical efficacy may be improved by developing methods of identifying tumour hypoxia, facilitating the selection of patients who will benefit most from hypoxia-modifying treatment.

14.6.1 Teaching an Old Drug New Tricks

Retrospective studies estimate that it takes an average of 15 years and over USD $1 billion to bring a single drug candidate onto the market. Traditional drug discovery using high-throughput screening (HTS) in a chemical space with over 10^{60} compounds is not only time-consuming, but also expensive. More alarmingly, new drug approvals by the US Food and Drug Administration (FDA) have plunged by 40 % since 2005 despite a concomitant doubling of investment in pharmaceutical research and development. This trend is unsustainable and there is an urgent need to solve the high rates of candidate drug attrition. One solution is to identify new uses for old drugs.

The Nobel laureate James Black famously once stated, "The most fruitful basis for the discovery of a new drug is to start with an old drug." In drug re-profiling, the molecular mechanisms of existing drugs are re-examined for novel therapeutic indications based on the concept of polypharmacology, where the average drug acts on five different targets (Overington et al. 2006). Screening of the John Hopkins Drug Library, a collection of small-molecules which have either been approved by the FDA or have entered phase II clinical trials, led to the serendipitous discovery of cardiac glycosides (Huafeng Zhang et al. 2008), anthracyclines (Lee et al. 2009a), and acriflavine (Lee et al. 2009) as inhibitors of HIF-1 synthesis, transcription, and dimerization respectively. Mitoxantrone was subsequently found to inhibit HIF-1α mRNA protein translation in a topoisomerase-II independent manner (Toh and Li 2011). In total, it is estimated that nearly 9,000 drugs are off-patent and available for such investigation. Critically, these drugs have known safety and pharmacokinetic profiles. This represents a significant cost advantage over traditional drug development, where more than 90 % of candidate molecules fail clinical testing due to safety concerns. They are also clinically used, enabling immediate commencement of phase II clinical trials for efficacy, and the move to clinic potentially rapid.

14.6.2 Targeted Drug Delivery

Nanopharmaceuticals are the next chapter in the fight against cancer. Early clinical trials with camptothecin – a potent dual inhibitor of topoisomerase I and HIF-1α – demonstrated high anti-tumour activity, but severe toxicity lead to discontinuation of the drug's development. This failed, but otherwise efficacious drug, may be rescued by shielding it in nanoparticles to improve its pharmacokinetics and safety profile. CRLX101 is a new nanopharmaceutical which contains a cyclodextrin-containing polymer conjugated to camptothecin. It is hypothesized that CRLX101 preferentially targets tumour cells by exploiting the large pores and fenestrations in tumour neovasculature. Sustained release of the drug, as opposed to a burst release followed by a rest period, also reduces the likelihood of developing drug resistance. In a combined phase 1/2 clinical trial, CRLX101 showed encouraging safety, pharmacokinetics, and efficacy – 64 % of patients treated at the maximum tolerated dose of 15 mg/m^2 bi-weekly achieved stable disease, with a median progression-free survival time of 3.7 months (Weiss et al. 2013). Phase 2 clinical development across multiple tumour types is ongoing.

Hypoxia-activated prodrugs are also highly desirable. The prodrugs are inactive when administered, and converted in a two-step process in vivo. Firstly, one-electron (1e) reduction of the relatively non-toxic prodrug creates an oxygen-sensitive intermediate. The intermediate undergoes spontaneous conversion into the active drug under hypoxic conditions, but is rapidly re-oxidized into the inert prodrug in the presence of oxygen. This futile redox cycle suppresses formation of the initial prodrug radical in oxic conditions, facilitating hypoxia-selective cytotoxicity. There are currently four classes of hypoxia-activated prodrugs in development, of which

the most promising candidate is TH-302 – a 2-nitroimidazole-triggered bromo analogue of the FDA approved DNA-alkylating agent ifosfamide. Initial studies of TH-302 with doxorubicin in advanced soft-tissue sarcoma demonstrated a high objective response rate, and a median overall survival (OS) of 17.5 months (Chawla et al. 2011). More recently, a randomized controlled trial involving 214 patients demonstrated a higher response rate to TH-302 in combination with gemcitabine as opposed to gemcitabine alone in advanced pancreatic cancer, with increases in median progression-free survival (PFS) of 5.5 months and 3.6 months respectively (Borad et al. 2012). Results from ongoing phase III trials in this setting are eagerly anticipated. Nevertheless, significant challenges remain in the development of hypoxia-activated prodrugs. One complicating factor is that many prodrugs may be activated by oxygen-insensitive two-electron (2e) oxidoreductases, resulting in cytotoxicity independent of hypoxia. Another key limitation is that hypoxic regions within tumours are frequently necrotic, and thus lack the enzymes and cofactors needed to reduce the prodrug. The use of hypoxia-regulated gene therapy is an intriguing solution to the problem. By engineering DNA constructs with hypoxia-responsive promoters, prodrug metabolizing enzymes may be selectively expressed at the tumour site (Dachs et al. 1997). However, delivery of vectors into solid tumours will be challenging. An alternative approach is to activate prodrugs using ionizing radiation. Radiolysis of water generates aquated electrons (e_{aq}^-), which may act as efficient reducing agents in lieu of endogenous enzymes. In addition, the radiation field may be focused onto the tumour to provide specificity in addition to hypoxia alone. This is a promising area of research to keep an eye on.

14.6.3 Patient Selection

Early clinical trials of tirapazamine – a hypoxia-activated prodrug – in advanced head and neck squamous cell carcinoma (HNSCC) demonstrated a significant decrease in locoregional failure versus 5-fluorouracil (5FU) combined with cisplatin and radiotherapy (Rischin et al. 2006). The subsequent phase III trial of tirapazamine, however, failed to meet its primary endpoints (Rischin et al. 2010). The key distinction between the two studies is that only the former used imaging to select for patients with evidence of tumour hypoxia. Recent meta-analyses of oxygen-electrode studies have further suggested that overall survival is compromised only in the subset of patients with extreme hypoxia (Nordsmark et al. 2005). Thus there is an urgent need to develop clinically applicable tools for the identification and quantification of tumour hypoxia.

Several imaging techniques are currently under development. In a study of 24 patients with prostate carcinoma, maps from blood oxygen level-dependent magnetic resonance imaging (BOLD MRI) yielded high sensitivity for defining hypoxic tumour regions stained with pimonidazole (Hoskin et al. 2007). However, it has limited specificity owing to influence from other factors including blood flow, CO_2, hematocrit, and pH. Hypoxic regions may also be identified using positron emission

tomography (PET) in combination with injectable tracers such as ^{18}F-MISO (Rasey et al. 1996). However, the technical complexities and limited availability behind these methods would likely preclude a wider clinical application.

The ideal biomarker should be accessible through non-invasive methods, be specific to hypoxia, and be sensitive to changes in pathology upon disease progression or therapeutic response. One candidate biomarker is osteopontin (OPN) – high concentrations of plasma osteopontin (OPN) are associated with tumour hypoxia as measured by Eppendorf electrodes, as well as decreased overall survival across multiple tumour types (Weber et al. 2010). In support of this hypothesis, retrospective analysis of 320 patients with squamous cell carcinoma of the head and neck from the DAHANCA 5 trial demonstrated that patients with high pre-treatment OPN levels benefited from hypoxic modification with nimorazole, but those with low to intermediate levels did not (Overgaard et al. 2005). However, plasma OPN was not shown to confer adverse prognosis or predict response to tirapazamine in the large TROG 02.02 phase III trial. Thus the clinical significance of plasma OPN is unclear. Approaches combining multiple biomarkers have been met with greater success. In a study of 323 HNSCC patients, classification of tumours as hypoxic based on the expression of a 15-gene hypoxia signature independently predicted benefit from the hypoxic radiosensitizer nimorazole (Toustrup et al. 2012). Validation is currently on-going.

There is also a need to stratify patients according to risk, so that interventions are focused on the population where hypoxia adversely affects treatment outcome. The molecular mechanisms of the hypoxic response of tumours are also dependent on tumour type and their respective microenvironments (Blouw et al. 2003). For example, HIF-1α inhibits the c-Myc oncoprotein to suppress tumour growth, whilst HIF-2α potentiates c-Myc transcriptional activity to accelerate tumour growth in renal cell carcinoma (Raval et al. 2005). By contrast, HIF-1α promotes tumorigenesis in the majority of human cancers. Nevertheless, hypoxia-modifying agents will prove advantageous in a select sub-group of patients.

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Chapter 15
Targeting Glycolytic Adaptations of Cancer Cells: From Molecular Mechanisms to Therapeutic Opportunities

Shanmugasundaram Ganapathy-Kanniappan

Abstract Metabolic reprogramming is one of the biochemical signatures of cancer cells. Particularly, aerobic glycolysis (i.e. the process of conversion of glucose into pyruvate followed by fermentation into lactate even in the presence of oxygen) has been of immense interest due to its impact not only on cancer cells but on the tumor microenvironment as well. Conceptual advancement in understanding the oncogenic regulation of glycolysis and multifunctional properties of glycolytic enzymes underscore the relevance and significance of targeting glycolysis in cancer cells. This chapter will discuss, in the light of recent research the intricacies of glycolytic adaptation in cancer cells, and the rationale for exploiting it for therapeutic intervention.

Keywords Glycolysis • Aerobic glycolysis • Metabolic reprogram • Acidosis • Chemoresistance • Microenvironment • Metabolic stress • ATP • Hypoxia • Apoptosis • Monocarboxylate transporters • Reactive oxygen species • Warburg effect • Embden–Meyerhof–Parnas pathway • Antiglycolytic therapy

15.1 Introduction

Cancer cells take up glucose vividly, and this metabolic phenotype is witnessed in most if not all solid tumors. This tumor specific change in glucose consumption is so ubiquitous in cancer that it has already been exploited in the clinical diagnosis of neoplasms, using the glucose analog, ^{18}F (fluoro)-2-deoxy glucose (FDG) by positron emission tomography (PET) imaging. A combined PET and computed tomography (CT) imaging could detect neoplasms with >90 % sensitivity and specificity

S. Ganapathy-Kanniappan, Ph.D. (✉)
Division of Interventional Radiology, Department of Radiology and Radiological Sciences, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Blalock 340, Baltimore, MD 21287, USA
e-mail: gshanmu1@jhmi.edu

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(Bomanji et al. 2001; Tang et al. 2011), which depends upon the viability and/or metabolic activity of cancer cells. The common and frequent occurrence of "increased utilization of glucose" by cancer cells also indicates the necessity or preference for such a biochemical alteration. Thus, glucose metabolism in general represents a potential and sensitive therapeutic target.

Intracellularly, glucose catabolism primarily occurs via one of the two major pathways. An energy-efficient but extended pathway that involves mitochondrial respiration (also known as oxidative phosphorylation) or a short but less energy efficient pathway (glycolysis) that does not depend on mitochondria. Depending upon the intracellular requirements and available resources (e.g. oxygen, nutrients) cells direct glucose metabolism either by oxidative phosphorylation or glycolysis. In the absence of sufficient levels of molecular oxygen, glucose catabolism does not occur via mitochondrial oxidation but rather through glycolysis resulting in the conversion of pyruvate into lactate which then can be exported. This process (under less oxygenated conditions) is referred to as "anaerobic glycolysis". Interestingly, in cancer cells glycolysis has been witnessed even in the presence of oxygen (hence referred to as "aerobic glycolysis"). The existence of an aerobic glycolytic phenotype in cancer cells has been known for almost a century since the seminal discovery by the German scientist Otto Warburg who proposed the "Warburg hypothesis" also known as the "Warburg effect" (Warburg et al. 1924). However, the causal factors and cancer-specific advantages of such altered metabolic phenotype remained obscure for several decades. Recent progress in understanding the regulation of energy metabolism has provided renewed impetus to explore the biological significance and clinical relevance of targeting tumor metabolism (Ganapathy-Kanniappan and Geschwind 2013). As a result, deregulated or altered energy metabolism has been recognized as one of the "hallmarks of cancer" (Hanahan and Weinberg 2011). Several elegant reviews have delineated a wealth of information on the biochemical processes of glycolysis and its biological significance with respect to tumor growth and poor prognosis (Gatenby and Gillies 2007; Pelicano et al. 2006). The objective of this chapter is to provide insights into the role of cellular stress in metabolic reprogramming, focusing particularly on glycolysis and its therapeutic potential as a drug target.

15.2 Tumor Glycolysis

Prior to the discussion of tumor glycolysis, in the interest of the readers, it is essential to understand its biochemical definition and the current usage in literature. In classical biochemistry, glycolysis *sensu stricto* refers to the conversion of glucose into pyruvate. Next, the conversion of pyruvate into lactate is known as fermentation. However, as indicated by several investigators, the process of conversion of glucose to pyruvate is common in both the lactate producing pathway as well as mitochondrial oxidation. This implies that irrespective of the mode of glucose oxidation, glycolysis will be an integral process. However, in cancer cells, as the difference between mitochondrial-dependent and independent pathways of glucose

utilization is linked to either inhibition or production of lactate, respectively, the term glycolysis from a cancer perspective is indicative of the conversion of glucose into lactate. Correspondingly, the non-glycolytic, oxidative phosphorylation refers to the conversion of glucose into pyruvate which will then be metabolized via the TCA cycle in mitochondria. Similarly, the most common type of glycolysis discussed elaborately in the literature is the Embden–Meyerhof–Parnas (EMP) pathway, named after its discoverers Gustav Embden, Otto Meyerhof, and Jakub Karol Parnas. However, modified processes of glycolysis are also known (e.g. Entner–Doudoroff pathway). For brevity and clarity, and also due to the relevance to tumor metabolism, the discussion here will be limited to the EMP pathway.

Uncontrolled proliferation and insensitivity to growth inhibitory signals result in the production of enormous biomass of cancer cells. Consequently, it is inevitable for a multicellular, three-dimensional tumor to be anatomically displaced from the primary source of blood supply. In this situation, cancer cells induce the formation of new blood vessels (neo-angiogenesis) to establish an alternative vascular network with existing vessels. However, due to incomplete or aberrant circuitry of capillaries cancer cells still remain under fluctuating levels of oxygen and/ or nutrients supply. Hence a metabolic switch from mitochondrial respiration to glycolysis under hypoxia and/ or mitochondrial dysfunction (Hu et al. 2012; Lu et al. 2012) is an adaptive mechanism necessitated to maintain uninterrupted growth of cancer cells. Nonetheless, a metabolic alteration to aerobic glycolysis under normoxic condition despite the presence of functionally competent mitochondria is intriguing. Furthermore, glycolysis is known to produce fewer adenosine triphosphate (ATP; the principal form of energy) molecules than oxidative phosphorylation per every molecule of glucose. Arguably, aerobic glycolysis will indeed increase the intracellular demand for ATP, a condition that will add metabolic stress. Yet, cancer cells of different tissue origin consistently exhibit an aerobic glycolytic phenotype. Until recently, understanding the biological rationale and cellular advantages of such metabolic shift remained a challenge. Teleological evidences demonstrate that a metabolic switch to glycolysis could provide selective advantage to cancer cells despite the low-yield in ATP (de Souza et al. 2011). For example, in order to minimize the difference in the production of total number of ATP due to the metabolic switch from mitochondrial respiration to glycolysis, cancer cells facilitate a higher rate of glycolysis. A higher glycolytic rate in turn elevates the rate of glucose oxidation into lactate as has been witnessed in cancer cells. This increase in the glycolytic rate thus maintains a faster rate of ATP production (Pfeiffer et al. 2001). In addition, such an elevated glycolytic rate has also been proposed to confer selective advantage under competition (between cancerous and non-cancerous (healthy) cells) for shared energy sources (Zhou et al. 2012). Some investigators opined that in cancer cells the bulk of the ATP pool is primarily required for cell maintenance rather than proliferation suggesting a minimal decrease in total number of ATP (due to the glycolytic switch) is not detrimental to cancer cells (Gatenby and Gillies 2004; Lunt and Vander Heiden 2011). Next, it is increasingly evident that glycolytic intermediates serve as precursors for the biosynthesis of macromolecules (e.g. NADPH and ribose-5-phosphate) which in turn are critical for cell growth (Deberardinis et al. 2008). In addition, the generation of NADPH via glycolysis facilitates the maintenance of

adequate levels of the antioxidant, reduced glutathione (GSH). GSH is indispensable not only for maintaining the redox balance but also to thwart the anticancer effects of some antineoplastic agents as well (Backos et al. 2012; Traverso et al. 2013). Thus aerobic glycolysis has been known to provide chemoresistance and resistance to radiotherapy (Pitroda et al. 2009). While aerobic glycolysis facilitates the pentose phosphate pathway (PPP) which in turn is critical for macromolecular biosynthesis, the PPP by itself has been known to render resistance to therapy as well (Riganti et al. 2012). Thus the cancer specific advantages of glycolysis could underly the preferential metabolic switch from mitochondrial oxidation to aerobic glycolysis.

15.3 Cellular Stress and Metabolic Reprogramming

In the words of Chi Dang, "metabolism generates oxygen radicals, which contribute to oncogenic mutations. Activated oncogenes and loss of tumor suppressors in turn alter metabolism" (Dang 2012). Cellular metabolic processes release several reactive molecules like hydrogen peroxide (H_2O_2), oxyradicals, hydroxyl (•OH) radicals etc., that are collectively known as reactive oxygen species (ROS). These ROS have been known to promote deleterious effects hence maintaining the cellular ROS level within threshold is critical for the maintenance of genomic integrity and cell survival. (Ray et al. 2012). Similarly, excessive accumulation of protons (H+) reduce the intracellular pH resulting in the disruption of normal physiology leading to cell death. Nevertheless, cells have evolved inherent mechanisms to respond to such undesirable changes in the levels of H+, ROS etc. For instance, intracellular levels of H+ are constantly maintained in homeostasis by proton exchange transporters by which protons are pumped out to the exterior. Similarly, the cellular response to mitigate the deleterious effects of oxidants (ROS) includes utilization of antioxidants (e.g. glutathione) which upon neutralization of the ROS becomes reduced.

In cancer cells in order to meet the constant demand for energy due to rapid proliferation and exponential growth, the rate of glucose utilization is elevated resulting in markedly high levels of glucose uptake. While the energy produced by high rate of glucose catabolism is necessary for cancer cell maintenance and growth, the by-products such as ROS released from mitochondrial oxidation will also raise to toxic levels. Though the redox balance is maintained by reduced-glutathione, an active antioxidant that neutralize enormous levels of ROS, a chronic elevation in the level of ROS will necessitate continuous replenishment of GSH. In this context, by diverting glucose catabolism away from mitochondrial respiration cancer cells can reduce the total ROS produced, decrease the protons (H+) released and more importantly conserve or minimize the utilization of available reducing equivalents (NADH) or antioxidants (GSH). Interestingly, glycolysis facilitates these desirable biochemical phenotypes besides providing several biological advantages, as discussed elsewhere. Although intracellular stress affects the overall physiology of cells, emerging data indicate that in cancer cells they do play a role in the promotion of altered tumor metabolism (Fig. 15.1). Among various stress molecules we will discuss ROS, intracellular-acidosis, and signaling mechanisms known to contribute to the metabolic switch to glycolysis.

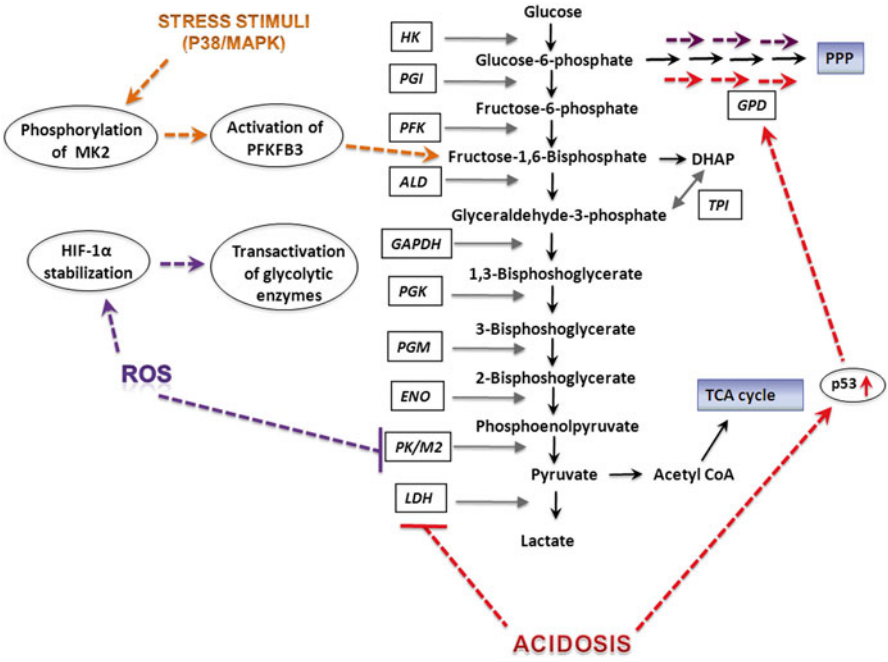

Fig. 15.1 Schematic showing the link between cellular stress and glycolysis. Stress stimuli trigger the stress signaling pathway such as p38/MAPK resulting in the activation of MAPK-M2 by phosphorylation. MAPK-M2 directly accelerates glycolysis by modulating PFKFB3 activity (indicated by *brown arrows*). Next, ROS, a major intracellular stress inducer also affects glycolysis by diverting glycolysis towards PPP (indicated by *purple arrows*). ROS affects the sulphydryl group of PKM2 resulting in its inactivation. Also, ROS promotes the stabilization of HIF1**α** which in turn activates several glycolytic enzymes resulting in an increase in the rate of glycolysis. Acidosis, another frequent and common intracellular stress, blocks LDH activity to redirect glucose oxidation via PPP (indicated by *red arrows*). Also, under acidosis p53 is up regulated which in turn promotes PPP by activating GPD. Enzymes are indicated in *square boxes* in *bold* and *italicized letters. Grey arrows* indicate the enzymes involved in glycolysis, *black arrows* indicate the sequential steps in glycolysis. *Purple color* refers to ROS mediated effects, *red color* refers to acidosis mediated effects and *brown color* refers to signaling mechanism mediated effects. *MAPK* Mitogen activated protein kinase, *PFKFB3* -phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase 3, *HIF1α* hypoxia inducible factor 1alpha (**α**), *PPP* pentose phosphate pathway, *ROS* reactive oxygen species, *HKII* hexokinase II, *PGI* phosphoglucose isomerase, *GPD* glucose -6-phosphate dehydrogenase, *PFK* phosphofructokinase, *ALD* aldolase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *TPI* triose phosphate isomerase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *ENO* enolase, *PKM2* pyruvate kinase M2 isoform, *LDH* lactate dehydrogenase, *PPP* pentose phosphate pathway, *TCA* cycle tricarboxylic acid cycle

15.3.1 ROS

It is widely known that excessive accumulation of ROS beyond cellular tolerance is cytotoxic. High rate of metabolism leads to an elevation in the level of cellular ROS creating an imbalance in the ratio of antioxidants and prooxidants. However, recent investigations have revealed that a minimal elevation in ROS prior to the

chronic/injurious level could provide cue to cancer cells for the necessity of metabolic alteration. In order to escape ROS mediated injury and mitigate further cellular stress alternative pathways such as glycolysis and PPP are facilitated (Sosa et al. 2013).

One of the adaptive mechanisms recently identified is that during high levels of ROS, the enzyme, purvate kinase-M2 isoform (PKM2) is inactivated by the modification of its suphydryl group by ROS. PK-M2 catalysis the conversion of phosphoenol pyruvate into pyruvate for further oxidation into either acetyl coA or lactate. Thus inactivation of PK-M2 affects the glycolytic step of pyruvate formation diverting it towards the PPP (Anastasiou et al. 2011). The advantage of this altered metabolism is that PPP generates NADPH which can rejuvenate oxidized glutathione into its reduced form to act as an active antioxidant. This way, glucose is diverted away from mitochondrial oxidation which while reducing the level of ROS produced, simultaneously supports the replenishment of glutathione to neutralize the cellular ROS level (Dang 2012). It is noteworthy that the PKM2 has also been indicated as a gatekeeper of cell growth and survival (Harris et al. 2012).

An intracellular increase in ROS levels also has been known to stabilize HIF-1alpha, a key protein which transactivates several genes of glycolysis (Semenza et al. 1994). Among the glycolytic enzymes that are up regulated, the activation of PDK results in the rewiring of the metabolic circuitry of glucose catabolism. PDK phosphorylates PDH resulting in its inhibition, directing the pyruvate to be converted to lactate by LDH. Similarly, the activation of PFKFB4 results in the degradation of 2,6-FBP (an activator of PFK1 that catalyzes the conversion of fructose 1 phosphate into fructose 1,6 bisphosphate, a rate limiting step of glycolysis) (Yalcin et al. 2009). Such inhibition of PFKFB4 has been known to redirect glucose into PPP (Ros and Schulze 2013). However, PFKFB3 is also activated by HIF-1alpha, and PFKFB3 drives glucose into glycolysis. Depending upon the cellular requirement particular isoforms of PFKFB (3 or 4) can play a critical role in the adopting the mode of glucose catabolism. Thus ROS influences energy metabolism by facilitating glucose utilization by non-mitochondrial pathways (glycolysis/PPP) enabling cancer cells to evade chronic intracellular stress.

15.3.2 Acidosis

Cellular acidosis in general can be defined as a decrease in intracellular pH that can affect normal cell physiology, eventually causing cell death. Lactic acid (lactate) generated by glycolysis may contribute to intracellular acidosis yet it is not an indispensable factor. A change in the level of intracellular H+ concentration is sufficient to cause acidosis. In an elegant report, by experimental manipulation of intracellular lactate levels and intracellular H+ concentration, Jen-Tsan Chi's group has demonstrated that acidosis can promote metabolic reprogramming (Lamonte et al. 2013). Under intracellular acidification, cancer cells favored the diversion of glycolysis into PPP (both oxidative and non-oxidative) and enhanced glutamine-metabolism to

meet biosynthetic (PPP) and bioenergetic (glutaminolysis) demands. It is also evident that acidosis also governs the oxidative and nonoxidative PPP depending upon the cellular requirements. Under nonoxidative PPP conditions (R5P-pentose phosphate), any excess or accumulation could be redirected or reversed back to glycolysis by the reversible reactions of transketolase and transaldolase.

Elevated levels of free protons (H+) are often shuttled to the extracellular tumor microenvironment to maintain intracellular pH (pHi) at physiologic levels. Increasing amounts of H+ being pumped into the extracellular space creates an acidic microenvironment, which is known to select for cells with enhanced metastatic potential as well as provide resistance to chemotherapy (Bailey et al. 2012; Moellering et al. 2008; Schlappack et al. 1991). Thus cellular response to intracellular pH enables metabolic reprogramming to evade cellular acidification-related cytotoxicity.

15.3.3 Signal Transduction

Stress responsive signal transduction mechanisms have been known to play a significant role in the regulation of several processes including cell cycle checkpoints, apoptosis etc. Mitogen activated protein kinase (MAPK) pathways (e.g. p38) account for many of such changes in cellular processes. However, evidence for any direct regulation of glycolysis by a signal transduction molecule remained elusive. Recently it has been demonstrated that MAPK-M2 (MAPK-activated protein kinase 2) activates PFKFB3, a key promoter of glycolysis (Novellasdemunt et al. 2013). Thus metabolic reprogramming to glycolysis is achieved through signaling pathways as well. Similarly, STAT1-dependent expressional regulation of glycolysis suggests a potential role for STAT1 as a transcriptional modulator of genes responsible for glycolysis (Pitroda et al. 2009). Thus increasing data indicate that a coordinated network facilitates the metabolic switch from oxidative phosphorylation to glycolysis. Taken together intracellular stress inducers including ROS, pH and others could influence redirection of glucose metabolism away from mitochondria but towards glycolysis and PPP.

15.4 Targeting Glycolysis

15.4.1 Rationale

Apart from providing the energy source, the intermediates (substrates/products) of glucose metabolism (glycolysis) are used for anabolic reactions as well. For example, glucose-6-phosphate is used for synthesis of ribose -5 phosphate for further use in nucleic acid synthesis, similarly, dihydroxyacetone for lipid synthesis. Multiple lines of evidence show that the enhanced glucose uptake witnessed in tumor cells is

to meet manifold requirements, and not just the energy demand. Thus, the enhanced glucose uptake is not just a favorite biochemical change, rather an indispensable metabolic transformation that is critical for the rapid, uncontrolled proliferation of tumor cells. In principle, targeting glucose metabolism essentially involves targeting more than one pathway that is interlinked with increased-glucose utilization.

Thus it is evident that aerobic glycolysis in conjunction with the PPP provide multiple benefits to cancer cells such as promoting tumor progression and providing resistance to therapy. Hence, this key signature of cancer cells, tumor metabolism, particularly the tumor glycolysis, provides an ideal target for therapeutic intervention.

Emerging data also substantiate several non-glycolytic functions of glycolytic enzymes and the metabolic intermediates of glycolysis (Fig. 15.2). Many enzymes of the glycolytic pathway such as hexokinase II (HKII), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK)-M2 isoform and lactate dehydrogenase (LDH) participate in a number of subcellular functions including gene regulation and histone modifications (Kim and Dang 2005). Similar to the enzymes of glycolysis, some of the metabolic intermediates of glycolysis are also involved in non-glycolytic functions. Fructose-1, 6 bisphosphate by maintaining cytochrome C in an inactive state plays an anti-apoptotic role in cancer (Diaz-Ruiz et al. 2008). While pyruvate, another metabolic intermediate of glycolysis, is known to promote chemoresistance by the upregulation of p-glycoprotein (Wartenberg et al. 2010) its transporters (monocarboxylate transporters, MCTs) regulate CD147, a matrix metalloproteinase inducer (Izumi et al. 2011; Pertega-Gomes et al. 2011). Taken together, these findings demonstrate that glycolytic enzymes and metabolic intermediates play a key role beyond glycolysis, impacting cancer cell growth.

It is noteworthy that a higher lactate level has been known to correlate with aggressive phenotype including tumor recurrence and the metastatic potential resulting in poor prognosis (Walenta et al. 2000). Since elevated lactate levels indicate the preponderance of glycolysis, antiglycolytic agents could be very effective in targeting such metabolic-phenotype in tumors. It is increasingly evident that lactate export mitigates intracellular acidification while its import into normoxic cancer cells provides a substrate source for TCA cycle (mitochondrial oxidation) and energy production. Thus a "metabolic symbiosis" prevails within a tumor due to the metabolic heterogeneity, viz. a central hypoxic and glycolytic population of cells, and a peripheral oxygenated tumor cells (Sonveaux et al. 2008). The existence of such a depending upon tumor vasculature and "give and take lactate" mechanism (Semenza 2008) will benefit both lactate-exporting and lactate-importing cells.

15.4.2 Therapeutic Opportunities

Oncogenic driver mutations have been known to culminate in altered signal transduction pathways enabling tumor cells to reprogram their metabolic circuitry to adapt to the microenvironment. For example, it has been demonstrated that enhanced nutrient uptake is an effect of oncogenic *RAS* mutations (Yun et al. 2009). The tumor-specific shift in metabolism has been shown to be inevitable for

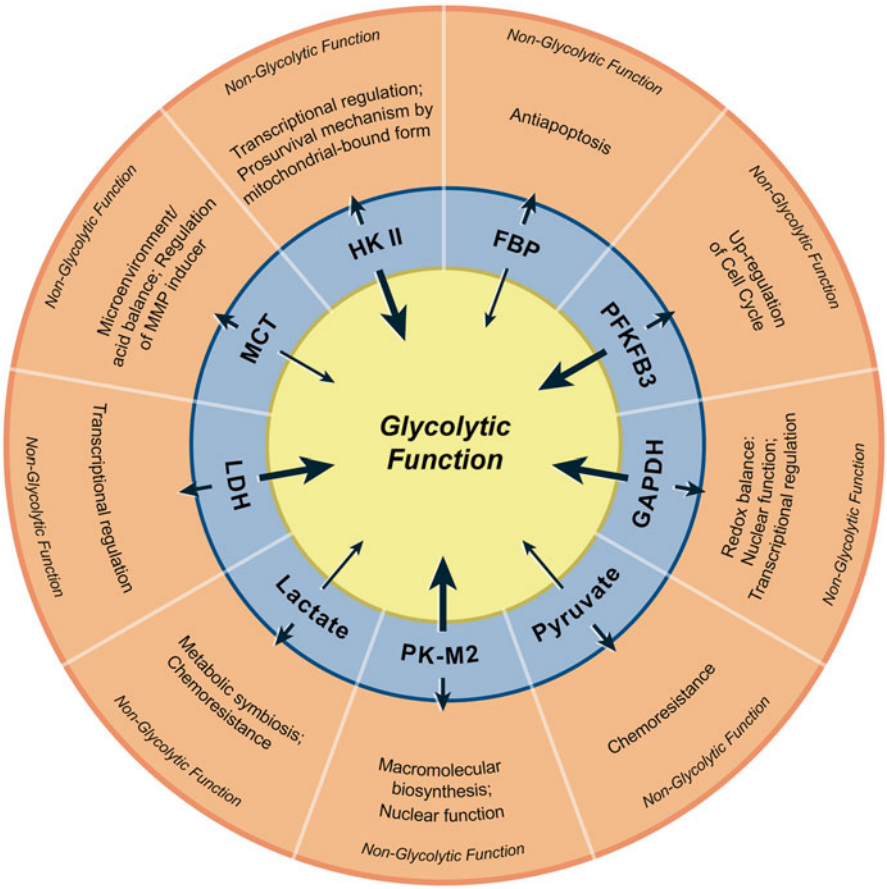

Fig. 15.2 Non-glycolytic functions of glycolytic enzymes and metabolic intermediates. In the innermost *circle*, *thick arrows* represent enzymes and *thin arrows* indicate intermediate metabolites. The *short arrows* pointing towards the *outer circle* represent the non-glycolytic functions of corresponding enzymes/metabolites. *HKII* hexokinase II, *FBP* fructose 1,6-bisphosphate, *PFKFB3* 6-phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase 3, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *PKM2* pyruvate kinase M2, *LDH* lactate dehydrogenase, *MCT* monocarboxylate transporters (Reproduced with permission from Molecular Cancer (2013))

uncontrolled proliferation and invasion of almost all solid tumors, hence the tumor metabolism is aptly described as "Cancer's Achilles' Heel" (Kroemer and Pouyssegur 2008). Thus, mounting evidence points to the notion that "enhanced or increased glucose uptake" of cancer cells could be a potential therapeutic target. Several reviews have emphasized and elegantly demonstrated the potential molecular targets of glycolysis that can be exploited for anticancer therapy. Figure 15.3 illustrates the biochemical steps that are blocked by currently explored inhibitors that are either under preclinical or clinical evaluation.

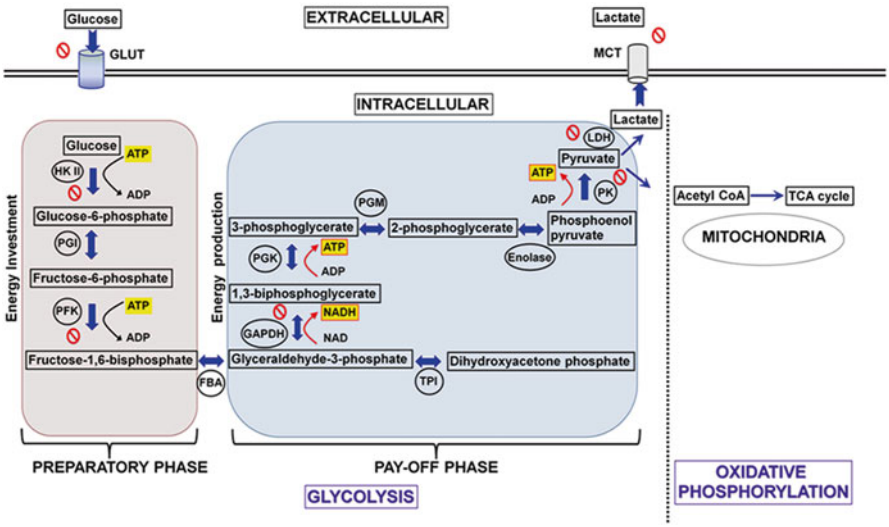

Fig. 15.3 Diagram showing the two phases of glycolysis and the molecular targets currently exploited for potential therapeutic drug strategies. Energy molecules such as ATP and NADH are highlighted in *yellow*, *black arrows* indicate consumption while *red arrows* indicate the energy release. The enzymes involved in respective reactions are abbreviated and encircled, where as the *block symbol* shows the targets exploited for drug development in preclinical investigations. *NADH* nicotinamide adenine dinucleotide reduced form, *GLUT* glucose transporters, *HKII* hexokinase II, *PGI* phosphoglucose isomerase, *PFK* phosphofructokinase, *FBA* fructose-bisphosphate aldolase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *TPI* triose phosphate isomerase, *PGK* phosphoglycerate kinase, *PGM* phosphoglycerate mutase, *PK* pyruvate kinase, *LDH* lactate dehydrogenase, *MCT* monocarboxylate transporter (Reproduced with permission from Molecular Cancer (2013))

15.4.3 Targeting Tumor and the Microenvironment

Several lines of evidence indicate that the impact of metabolic reprogramming to glycolysis is not confined to cancer cells but extends to the stroma/fibroblasts in the tumor microenvironment which favors tumor progression by sustained fuel or energy supply. These microenvironment-glycolytic reprogramming are also orchestrated by oncogenes. It is important to recall that oncogenetic activation (RAS, NF-kB etc.) and tumor suppressor loss have also been shown to facilitate the metabolic reprogramming of tumor microenvironment. Thus, the cancer cell's metabolic reprogramming to glycolysis also directs the metabolic reprogramming of cancer associated fibroblasts (Lisanti et al. 2013). It has been known that tumor microenvironment acts as a barrier and renders defense against therapeutic agents. Targeting tumor glycolysis will therefore affect the tumor microenvironment which could disrupt microenvironment-related protumorigenic properties.

15.4.3.1 Combination with Chemotherapy/Radiation Therapy

Clinical outcome of chemotherapeutics thus far clearly demonstrate that a monotherapy may not be as effective as combination therapy. If under a personalized medicine approach, combinatorial strategy is required antiglycolytic agents might be a better candidate to promote potent anticancer effects. Thus antiglycolytic agents may provide an additional line of attack via combination therapy. Such approaches have already been evaluated in preclinical models to overcome drug resistance in cancer (Dwarakanath and Jain 2009; Maschek et al. 2004). As discussed earlier, since glycolysis also plays a pivotal role in resistance to therapy its inhibition potentially could sensitize tumor cells for any chemotherapy. Therefore, the combinatorial therapeutic approach with antiglycolytic agents could be a vital strategy against resistant phenotypes.

Under radiation therapy, cancer cells induce aerobic glycolysis through reactive oxygen species. Reports have identified the Warburg effect to be implicated in resistance to cytotoxic stress, including ionizing radiation as well as chemotherapy. Therefore, treatment methods which block or reduce glycolytic metabolism after radiotherapy may increase tumor cell sensitivity to radiation and chemotherapeutic killing (Zhong et al. 2013).

15.4.3.2 Antiglycolytic Strategy and Induction of Immune Response

The ability to evade immune surveillance is one of the hallmarks of cancer, and it has been well established that tumor cells escape immune detection through immunosuppressive networks. One of the factors that challenge the functional efficiency of antitumor-immune cells is the maintenance of a relatively low pH in the tumor micro environment. Tumors achieve this by regulating lactic acid secretion via modification of glucose/glutamine metabolism. Cancer-generated lactic acid could thus be viewed as a critical, immunosuppressive metabolite in the tumour microenvironment rather than a ‘metabolic waste product’ (Choi et al. 2013). Thus antiglycolytic therapy should preferably reduce or prevent lactate accumulation which in turn could promote or elicit the host immune response. The inhibition of glycolysis followed by alteration in the microenvironmental lactate levels could expose cancer cells as vulnerable to host immune surveillance, providing an opportunity for immunotherapy (Beneteau et al. 2012).

15.5 Summary

In summary, substantial evidences establish the scientific rationale for targeting glycolysis in cancer cells. Aerobic glycolysis indeed is an integral component of the altered metabolism of cancer, a hallmark that has received renewed interest in the

recent decades. Several candidate drugs have been developed and evaluated mostly at the preclinical level with mixed success. Selective candidates (e.g. 2-deoxyglucose) have entered clinical trials, yet their translation remains to be witnessed. Mechanistically, the therapeutic potential of antiglycolytic strategy also includes the activation of proapoptotic pathways that are deregulated in cancer cells. Aerobic glycolysis suppresses p53 activity in cancer cells to provide selective protection from apoptosis upon loss of growth signal or inhibition of BCR-Abl (Mason et al. 2010). Thus inhibition of glycolysis could eliminate the antiapoptotic status of p53 resulting in the induction of tumor cell death. Similarly, it is also suggested that inhibition of glycolysis could sensitize cancer cells to AMPK activator-dependent induction of apoptosis (Pradelli et al. 2010). Therefore, molecular targeting of glycolysis could promote a myriad of effects including sensitization to chemotherapy and radiation therapy, and activation of apoptotic mechanisms in addition to the primary effect of disruption of energy metabolism. Up till now the major impediment for the successful clinical translation of any potent antiglycolytic agents is the manifestation of undesirable systemic toxicities which emanate from non-specific targeting. Future investigations to design and develop antiglycolytic agents that are selective in targeting cancer cells could greatly improve the therapeutic opportunities in the fight against cancer. To conclude, as the link between altered energy metabolism and cancer is increasingly evident targeting the metabolic reprogramming such as aerobic glycolysis could be an effective strategy for successful cancer therapy.

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Chapter 16
At the Crossroads Between Mitochondrial Metabolite Transport and Apoptosis: VDAC1 as an Emerging Cancer Drug Target

Varda Shoshan-Barmatz, Anna Shteinfer, Danya Ben-Hail, Tasleem Arif, and Dario Mizrachi

Abstract Many cancer cells undergo re-programing of metabolism and develop cell survival strategies involving anti-apoptotic defense mechanisms, a hallmark of a great majority of cancer types. The voltage-dependent anion channel 1 (VDAC1), an outer mitochondria membrane protein, serves as a mitochondrial gatekeeper, controlling the metabolic and energy cross-talk between mitochondria and the rest of the cell. VDAC1 has also been recognized as a key protein in mitochondria-mediated apoptosis due to its association with pro- and anti-apoptotic members of the Bcl-2 family of proteins. At the same time, VDAC1 functions in the release of apoptotic proteins located in the inter-membranal space. Thus, VDAC1 is emerging as an excellent target for impairing the re-programed metabolism of cancer cells and their ability to evade apoptosis. Here, we review current evidence pointing to the function of VDAC1 in cell life and death, and highlight these functions in relation to cancer therapy. We discuss the use of VDAC1-based strategies to attack the altered metabolism and apoptosis of cancer cells. These strategies include specific siRNA to impair energy and metabolic homeostasis, leading to arrest cancer cell growth and tumor development, as well as VDAC1-based peptides interacting with anti-apoptotic proteins and inducing apoptosis, thereby overcoming the resistance of cancer cell to chemotherapy. Finally, small molecules targeting VDAC1 can induce apoptosis. VDAC1 can thus be considered as standing at the crossroads between mitochondrial metabolite transport and apoptosis and hence represents an emerging cancer drug target.

Keywords Bcl-2 • Bcl-xL • ATP • Metabolic homeostasis • Cancer • Metabolic reprogramming • Hexokinase • Mitochondria • Oligomerization • Peptides • siRNA

V. Shoshan-Barmatz (✉) • A. Shteinfer • D. Ben-Hail • T. Arif
Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
e-mail: vardasb@bgu.ac.il

D. Mizrachi
School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14850, USA

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• VDAC • VDAC isoforms • VDAC-based peptides • ROS • Cholesterol transport • Warburg effect

Abbreviations

AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocase
APAF-1	Apoptosis protease-activating factor 1
Bcl-2	B-cell lymphoma 2
CLL	Chronic lymphocytic leukemia
CyP D	Cyclophilin D
Cyto *c*	Cytochrome c
HK	Hexokinase
IMM	Inner mitochondrial membrane
MAC	Mitochondrial apoptosis-induced channel
OMM	Outer mitochondrial membrane
PLB	Planar lipid bilayer
PTP	Permeability transition pore
ROS	Reactive oxygen species
STS	Staurosporine
TSPO	Translocator protein
VDAC	Voltage-dependent anion channel

16.1 Introduction

16.1.1 Overview

In recent years it has become evident that mitochondria play a major role in different events beyond their critical bioenergetics function of supplying ATP, such as in cell signaling events, inter-organellar communication, aging, cell proliferation, disease, and apoptosis (programmed cell death). It is paradoxical that mitochondria, which are indispensable for cell survival, are also necessary for suicidal cell death. Apoptosis is believed to eliminate cells whose metabolism and genomic organization have undergone transformations that may lead to malignancy. Thus, apoptosis is one of the main natural mechanisms protecting against cancer development. However, genetic alterations drive malignant cell protection from apoptosis, a hallmark of the majority of cancer types, and the most frequent cause of treatment failure.

One of the mitochondrial proteins controlling cell life and death is the voltage-dependent anion channel 1 (VDAC1), also known as mitochondrial porin. Recently, VDAC1 has been identified as a dynamic regulator of global mitochondrial function both in health and disease (Lemasters and Holmuhamedov 2006; Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz and Mizrachi 2012).

The focus of this review will be on the central role of VDAC1 in cell life and death, with an emphasis on current approaches for targeting the multi-functional behavior of VDAC1 in cancer cells as new strategies for inhibiting cancer cell growth or to overcome their mechanisms of protection from apoptosis.

16.1.2 VDAC1: Structure, Conductance, Ion Selectivity and Voltage-Dependent Gating of the Reconstituted Channel

16.1.2.1 VDAC1 Structure

Various studies have led to the development of membrane topology models postulating the trans-membrane organization of VDAC1, comprising a single α-helix at the N-terminus and 13, 16 or 19 trans-bilayer β–strands that together form a β-barrel (Shoshan-Barmatz and Mizrachi 2012). However, recent 3D-structures of recombinant VDAC1 obtained using X-ray crystallography, NMR or a combination of both reported VDAC1 to contain 19 β-strands arranged as a barrel, with strands β1 and β19 being in parallel conformation (Fig. 16.1A, B) (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008). All three methods employed refolded recombinant VDAC1 expressed in *E. coli*, purified from inclusion bodies. As such, it has been argued that the refolding conditions employed led to the appearance of non-native structures, as biochemical and biophysical approaches argue for the existence of additional extra-membranal VDAC1 regions (Colombini 2009). These structural studies further suggested that the N-terminal region of VDAC1, consisting of 25 amino acids, lies inside the channel pore and possesses different degrees of α-helical content in each of the three proposed structures (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008). The pore diameter of the channel has been estimated to be between 3 and 3.8 nm, based on biochemical and structural methods (Bayrhuber et al. 2008) and about 1.5 nm when the N-terminal α-helix is located within the pore (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008). However, the N-terminal domain was shown to be highly dynamic and to translocate out of the pore (Geula et al. 2012a; Shoshan-Barmatz et al. 2013) (Fig. 16.1Ac). It was proposed that the α-helix structure is involved in the positioning of this domain within the pore and that the glycine-rich ^{21}GYGFG^{25} sequence provides the flexibility required for N-terminal region translocation out of the channel internal pore (Geula et al. 2012a). N-terminal region mobility is further supported by observations that this protein segment exhibits motion during voltage gating (for more details see (Geula et al. 2012a)), that anti-VDAC1 antibodies raised against the N-terminal region of the protein interact with membranal VDAC1 (Abu-Hamad et al. 2006), and that it mediates the interaction of VDAC1 with the anti-apoptotic and pro-survival factors, hexokinase (HK)-I, HK-II and Bcl-2, suggesting its exposure out of the pore (Abu-Hamad et al. 2009). A VDAC1 dimeric structure was also demonstrated (Fig. 16.1Ad) (Bayrhuber et al. 2008).

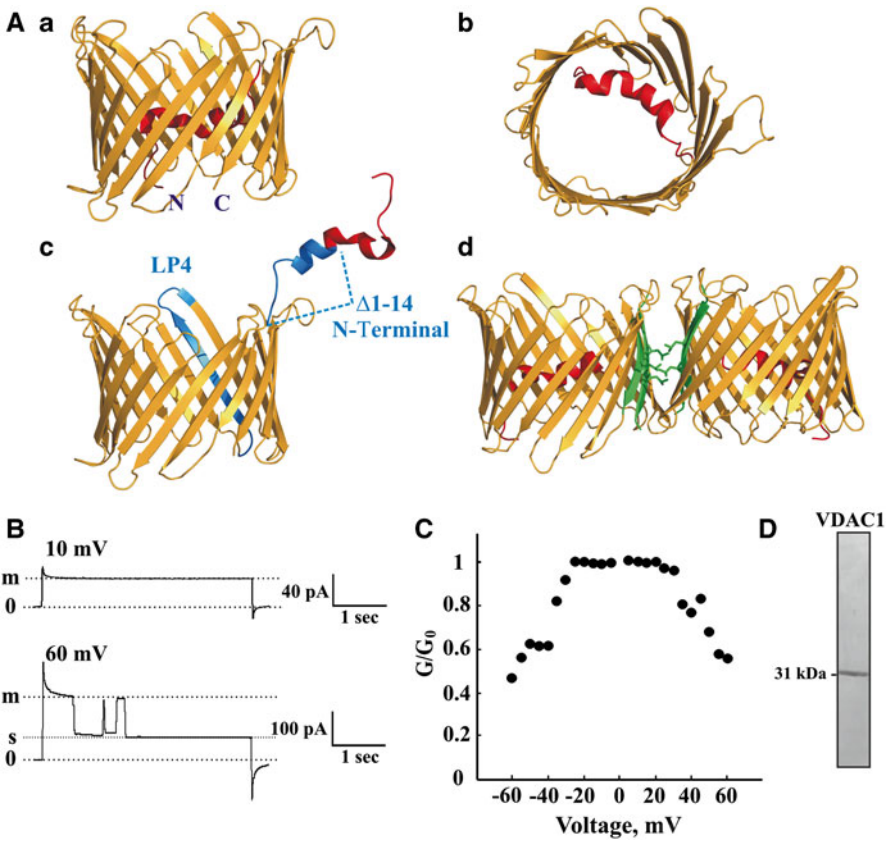

Fig. 16.1 Three-dimensional structure of VDAC1 and its channel activity. (**A**), VDAC1 monomer and dimer structures: (**a**) *Side view* of the crystal structure of VDAC1 (PDB code: 3EMN). The β-barrel is formed by 19 β-strands and the N-terminal helix is folded into the pore interior. Both the N- and C-termini (N and C, respectively) are located on the same side of the membrane. The N-terminus is colored red. (**b**) *Top view* of VDAC1 with the N-terminal helix nested inside the VDAC1 pore. (**c**) A proposed model for the conformation of VDAC1 with its N-terminal on the outside of the VDAC1 pore. The LP4 and (ΔN1-14)N-Terminal domains used as anti-cancer peptides are depicted in *blue* (see Sect. 1.7). (**d**) A proposed dimer of VDAC1. Figures were prepared using PyMOL software. (**B**, **C**) Bilayer-reconstituted VDAC1 single and multi-channel activity was assayed as described previously (Arbel et al. 2012). (**B**) shows a typical current recording through VDAC1 (1 M NaCl) in response to voltage steps from 0 to 10 mV showing a constant conductance, and from 0 to 60 mV, with the current first increased (m) due to a greater driving force then decreased due to a conformational change to a low-conducting state (s). (**C**) The average steady-state conductance of VDAC1 is presented as a function of voltage. Relative conductance was determined as the ratio of conductance at a given voltage (G) and the maximal conductance (G_0) at 10 mV, showing the bell-shape voltage-dependence characteristic of VDAC1. (**D**) Mitochondria-purified VDAC1 that was used in the PLB experiments

16.1.2.2 Channel Properties

The channel properties of purified VDAC1 have been examined following reconstitution of the purified protein into a planar lipid bilayer (PLB), using various procedures and detergents (Shoshan-Barmatz et al. 2010a). Bilayer-reconstituted VDAC1 assumes multiple voltage-dependent conformational states (Fig. 16.1B) displaying different selectivities and permeabilities. VDAC1 shows symmetrical bell-shaped voltage-dependent conductance (Fig. 16.1C) with the highest conductance (4 nS at 1 M KCl) occurring at low potentials of −20 to +20 mV (Colombini 2012). At low potentials, and when in the fully open state, VDAC1 selectively conducts small ions (e.g. Cl^-, K^+, Na^+), yet shows a preference for anions, such as phosphate, chloride, adenine nucleotides, glutamate, and other anionic metabolites, and large cations, such as acetylcholine, dopamine and Tris (Shoshan-Barmatz et al. 2010a). At higher positive or negative potentials (>30–60 mV), channel conductance is reduced and the selectivity shifts to small cations. In this scenario, the channel becomes virtually impermeable to ATP and ADP (Colombini 2012; Shoshan-Barmatz et al. 2010a).

As a voltage-gated channel, it is believed that VDAC1 channels rely on two separate gating processes, one at positive trans-membrane potentials and the other at negative potentials (Colombini 2012; Shoshan-Barmatz et al. 2010a). The N-terminal α-helical segment of the channel has been proposed to act as the voltage sensor, gating the pore via conformational changes and/or movements (Sect. 1.2.1). Clearly, additional studies are required before the molecular nature of the VDAC1 gating mechanism can be resolved.

16.1.3 VDAC Functions Are Important for Cancer Cells

VDAC is a 31 kDa pore-forming protein found in the outer mitochondrial membrane (OMM) of all eukaryotes (Colombini 2012; Shoshan-Barmatz et al. 2010a). Three eukaryotic VDAC isoforms, encoded by three separate genes sharing 65–70 % of sequence homology, VDAC1, VDAC2 and VDAC3, have been identified (for review, see (Shoshan-Barmatz et al. 2010a)). All VDAC isoforms can be found in most tissues, albeit at different levels of expression, with the most abundant and studied being VDAC1 (Shoshan-Barmatz et al. 2010a). The specific role of each isoform remains unclear, although evidence indicates that the three isoforms may serve different physiologic functions (Shoshan-Barmatz et al. 2010a). The focus of this review is on VDAC1.

16.1.3.1 VDAC1 Transport Activity Can Control the Fate of the Cell

At the OMM, VDAC1 functions as gatekeeper for the entry and exit of mitochondrial metabolites, assuming a crucial position in the cell, serving as the main interface between mitochondrial and cellular metabolisms. VDAC1 thus mediates the fluxes of ions, nucleotides and other metabolites up to ~5,000 Da across the OMM (Shoshan-Barmatz et al. 2010a) (Fig. 16.2A). Moreover, its location at the

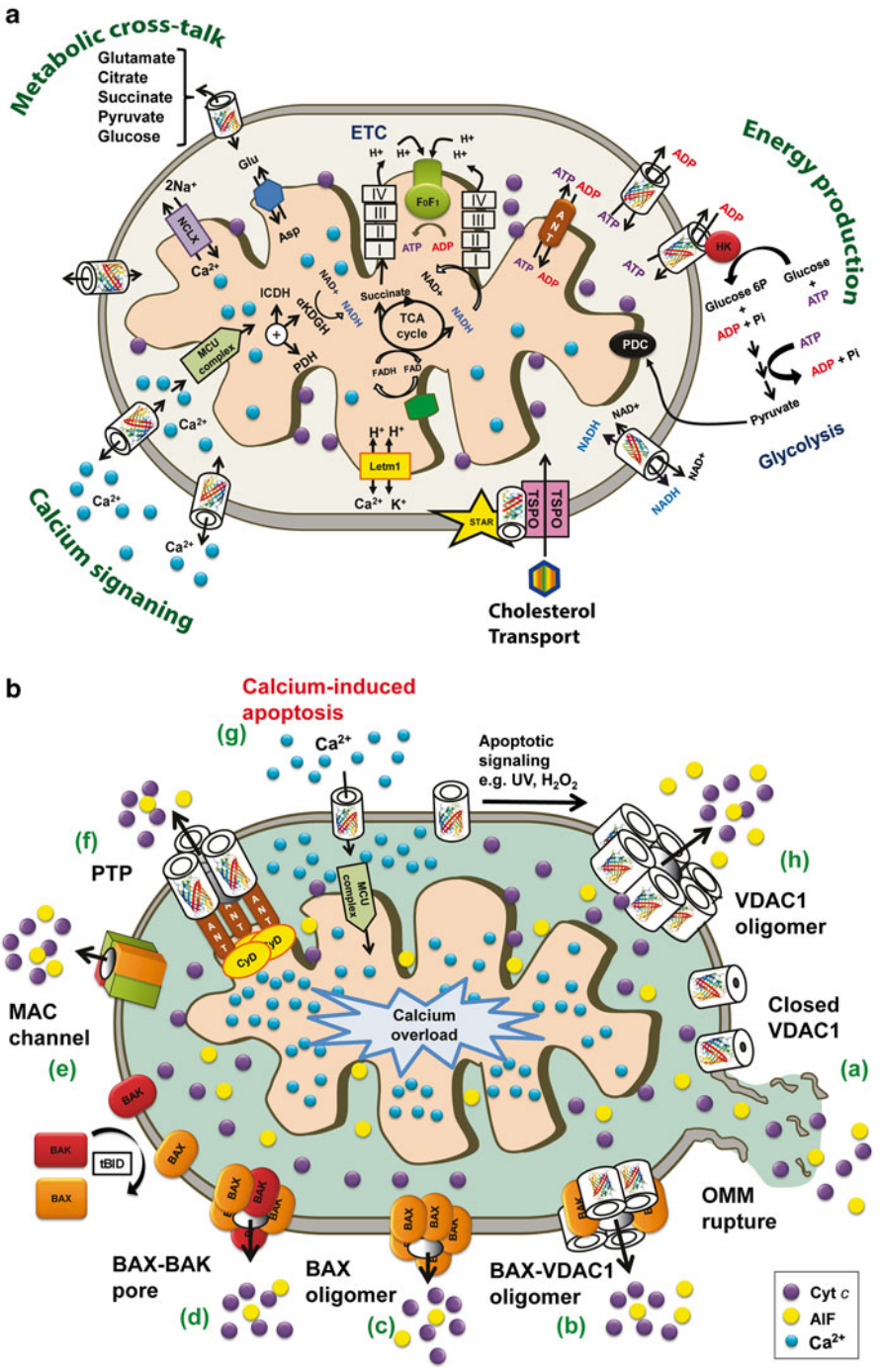

Fig. 16.2 Schematic representation of VDAC1 as a multi-functional channel essential for cancer cell survival and regulator of cell death. (**A**) VDAC1 functions in cell life – The various functions of VDAC1 include control of the metabolic cross-talk between the mitochondria and the rest of the cell, cellular energy production by transporting ATP/ADP and NADH between the

boundary between the mitochondria and the cytosol enables VDAC1 to interact with proteins that mediate and regulate the integration of mitochondrial functions with other cellular activities (Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Mizrachi 2012).

VDAC1 Transport Activity Controls Energy and Metabolites

In the open state, VDAC1 allows free shuttling of ATP and ADP as well as NAD+/NADH. Mitochondria-generated ATP is transported to the cytosol for exchange with ADP, which is utilized in oxidative phosphorylation to generate ATP. As such, VDAC1 controls the electron transport chain, helping to generate energy and support survival (Shoshan-Barmatz et al. 2010a). VDAC1 closure limits the normal flow of metabolites in and out of mitochondria and thus impairs cellular metabolic and energy homeostasis (Vander Heiden et al. 2000). Indeed, silencing VDAC1 expression resulted in reduced metabolite exchange between mitochondria and the cytosol, showing VDAC1 to be essential for energy production and cell growth (Abu-Hamad et al. 2006; Arif et al. 2014).

VDAC1 function in energy metabolism is also reflected in its interaction with hexokinase (HK) and creatine kinase (CK) to convert newly generated ATP into high-energy storage forms, such as glucose-6-phosphate and creatine phosphate, respectively (Shoshan-Barmatz et al. 2010a). In keeping with its two-way trafficking role, VDAC1 also enables the exit of newly formed hemes, as well as the entrance of substrates of the electron transport chain, including pyruvate, malate, succinate and NADH (Shoshan-Barmatz et al. 2010a).

Fig. 16.2 (contiuned) inter-membrane space (IMS) and the cytosol, binding HK, Ca^{2+} signaling by transporting Ca^{2+}, and cholesterol transport. Also presented are the Ca^{2+} influx and efflux transport systems of the OMM and IMM, as well as Ca^{2+}-mediated regulation of the tricarboxylic acid (TCA) cycle via activation of pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH) and α-ketoglutarate dehydrogenase (α KGDH). The electron transport chain (ETC) and the ATP synthase (F_oF_1) are also presented. VDAC1 in the OMM transports Ca^{2+} to the IMS. The uptake of Ca^{2+} into the matrix via the IMM is mediated by the mitochondrial Ca^{2+} uniporter (MCU), regulated by a calcium-sensing accessory subunit (MICU1). Ca^{2+} efflux is mediated by NCLX, a Na^+/Ca^{2+} exchanger. High levels of matrix Ca^{2+} accumulation trigger the opening of the permeability transition pore (PTP), a fast Ca^{2+} release channel. (**B**) VDAC1 function in cell death – Different models for the release of apoptogenic proteins, such as Cyto *c* (*purple circles*) and AIF (*yellow circles*) from the IMS to the cytosol, leading to apoptosis. These models include: (**a**) VDAC1 closure and OMM rupture serving as the Cyto *c* release pathway; (**b**) A Bax- and VDAC1-based hetro-oligomer mediating Cyto *c* release; (**c**) Bax activation followed by its oligomerization resulting in OMM permeabilization; (**d**) A pore formed by oligomerized forms of Bax and Bak; (**e**) MAC forms during the early apoptosis stage as the release pathway; (**f**) A PTP composed of VDAC1 at the OMM, ANT at the IMM and CypD in the matrix providing the apoptogenic protein release pathway; (**g**) Mitochondrial Ca^{2+} overload induces apoptosis – Ca^{2+} transport across the OMM, as mediated by VDAC1, and then across the IMM, as mediated by the MCU, leads to Ca^{2+} overload in the matrix. This is turn causes dissipation of the membrane potential, mitochondria swelling, PTP opening, Cyto *c* release and the triggering of apoptotic cell death; (**h**) A VDAC1 homo-oligomer forming the apoptotic proteins-conducting channel (see Sect. 1.5)

VDAC1 in Cholesterol Transport

In general, cancer cells have been shown to exhibit two- to ten-fold more mitochondrial cholesterol (mainly in the OMM) than found in liver mitochondria, thus altering the fluidity of the cancer cell membrane (Yu et al. 2005). Cholesterol is transported across the OMM (Fig. 16.2A) (Rone et al. 2009), with VDAC1 being considered to be a necessary component of a multi-protein complex, the transduceosome, thought to also contain the high-affinity cholesterol-binding protein translocator protein (TSPO) and the steroidogenic acute regulatory protein (STAR) (Campbell and Chan 2008). TSPO interacts with VDAC1, helping to anchor the multi-protein complex to the OMM and assists with the binding and import of STAR (Miller 2013), hence serving as the acute regulator of steroidogenesis. In addition, high cholesterol affects HK binding to VDAC1, and accordingly, the metabolic function of VDAC1 (Campbell and Chan 2008). Thus, VDAC1 affects both cholesterol synthesis and transport, and is subject to cholesterol-mediated regulation.

VDAC1 as a ROS Transporter

Reactive oxygen species (ROS) are well-known to play a part in proliferation and cell death, with cellular levels of ROS being linked to anti-tumor immunity, the oxidative tumor micro-environment, the proliferation and death of cancer cells (Manda and Neagu 2009). Hypoxia, a characteristic of most solid tumor micro-environments, causes a progressive elevation in mitochondrial ROS production (chronic ROS) which activates the transcription of genes involved in cellular hypoxic adaptation (Hamanaka and Chandel 2009).

Mitochondria is a major source of ROS in the cell that attacks DNA, lipids and proteins, thereby affecting cell survival (Handy and Loscalzo 2012). ROS release to the cytosol is mediated by VDAC1, with such transport being regulated by VDAC1-bound HK-I and HK-II, serving to reduce intracellular levels of ROS (Fig. 16.2B) (da-Silva et al. 2004). Closure of VDAC1 causes oxidative stress and accelerates Ca^{2+}-induced opening of the mitochondrial permeability transition pore (PTP) (Tikunov et al. 2010). As mitochondrial dysfunction is one of the important features of ROS-mediated cell death, the ROS release function of VDAC1 is an important activity protecting against mitochondrial damage.

In summary, VDAC1 appears to be a convergence point for a variety of cell survival signals regulating the metabolic and energetic functions of mitochondria by its transport activity, as well as by its association with various ligands and proteins. As such, VDAC1 can control the fate of cancer cells (Abu-Hamad et al. 2009; Abu-Hamad et al. 2006).

16.1.4 Cancer Cell Metabolism and Targeting VDAC1 by siRNA to Inhibit Cell and Tumor Growth

In recent years, a substantial body of evidence has accumulated indicating a correlation between alterations in cell metabolism and cancer formation (Gatenby and Gillies 2004; Hanahan and Weinberg 2011; Koppenol et al. 2011; Shoshan-Barmatz and Golan 2010). Cancer cells undergo significant metabolic adaptation to fuel cell growth and division (Gatenby and Gillies 2004; Hanahan and Weinberg 2011; Koppenol et al. 2011). Malignant cancer cells typically display high rates of glycolysis even when fully oxygenated and are subject to suppressed mitochondrial respiration, despite the fact that glycolysis is a less energy-efficient pathway, a phenomenon known as the 'Warburg effect' (Gatenby and Gillies 2004; Hanahan and Weinberg 2011; Koppenol et al. 2011). The Warburg effect likely provides the vast majority of cancerous cells with a number of benefits in the form of precursors for the biosynthesis of nucleic acids, phospholipids, fatty acids, cholesterol and porphyrins. A second advantage of the Warburg effect is its expected involvement in both tumor protection and invasion. Tumor cells produce lactic acid via glycolysis and transport it out of the cell, leading to increased acidity of the closed micro-environment and the generation of a low pH 'coat'. This coat is proposed to protect tumors against attack by the immune system while inducing negative effects on normal surrounding cells, aiding in preparing the surrounding tissues for invasion. Additionally, the Warburg effect also assures longer tumor survival time if oxygen becomes limiting (Hanahan and Weinberg 2011). Moreover, cancer-associated abnormalities in glucose metabolism enhance cellular resistance to apoptosis, with mitochondria playing a key role in this process (Fulda et al. 2010; Gogvadze et al. 2010; Kroemer and Pouyssegur 2008; Mayevsky 2009). Finally, mitochondria have been found to contribute to cellular re-programming from the catabolic to the anabolic mode (Gogvadze et al. 2010; Kroemer and Pouyssegur 2008; Mayevsky 2009), with such metabolic flexibility and cellular hierarchy being crucial in metastatic cancer (Berridge et al. 2010).

VDAC1 functions are indispensable for proper mitochondria functions and, consequently, for cell activity. Specifically, VDAC1 is crucial for a range of cellular processes, including ATP rationing, Ca^{2+} homeostasis and apoptosis execution (Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz and Mizrachi 2012 (Fig. 16.2B). These activities are regulated via the interaction of VDAC1 with many proteins central to the regulation of cell survival and cellular death pathways (Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz and Mizrachi 2012). VDAC1 serves as a key binding target for nearly two dozen proteins (see Sect. 1.6), (Hanahan and Weinberg 2011; Shoshan-Barmatz et al. 2006; Shoshan-Barmatz and Mizrachi 2012). These interactions point to VDAC1 as a convergence point for a variety of cell survival and cell death signals, and thus interfering with these interactions could impair cell homeostasis, as desired in case of cancer cells.

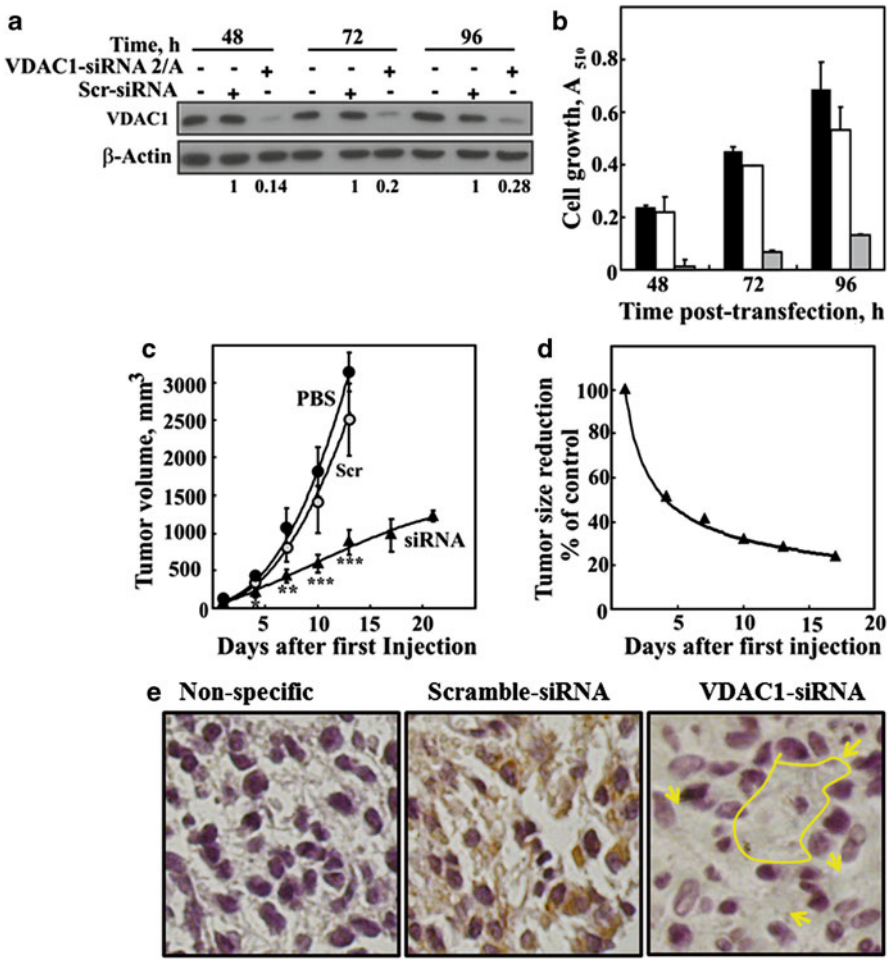

Fig. 16.3 Modified hVDAC1-siRNA-mediated inhibition of cell growth *in vitro* and *in vivo*. U87MG cells were transfected with 50 nM siRNA-hVDAC1 or scrambled siRNA and VDAC1 expression levels (relative units, RU) were evaluated by immunoblot at 48, 72 and 96 h post-transfection (**a**). Similarly, cell growth was assayed using the SRB method (**b**), with *black*, *grey* and *white bars* representing non-transfected cells and cells transfected with scrambled or hVDAC1-siRNA, respectively (n = 3). In a xenograft mouse model (**c**, **d**), U87MG cells were inoculated into male nude mice (2×10^6 cells/mouse). Tumor volumes were monitored (using a digital caliper) and on day 20, the mice were divided into three groups (eight or nine mice per group), with each group containing a similar average tumor volume (120 mm^3). The three mice groups were subjected to the following treatments. Xenografts were injected at two points with PBS (●, control), with scrambled siRNA (o) or with VDAC1-siRNA (▲) (10 µl of a 400 nM solution to yield a final concentration of 40–60 nM of each siRNA). Xenograft sizes as a function of time following the start of treatment is presented in (**c**), while the fold decrease in tumor size of the VDAC1-siRNA-injected xenografts, as compared to PBS-injected mice, is shown in (**d**). The calculated average tumor volumes are presented as means ± SEM, $P < 0.01$ (**) or < 0.001 (***). (**e**) Histological analysis of paraffin sections cut from tumors removed from scrambled- and VDAC1-siRNA-treated mice was carried out by hematoxylin/eosin and immunohistochemical staining with anti-VDAC1 antibodies, recognizing both mouse

16.1.4.1 Silencing VDAC1 Expression by siRNA Inhibits Cancer Cell Proliferation and Tumor Growth *In Vivo*

The over-expression of VDAC1 in some cancer cells may be related to its multi-functional activities required by the high-energy demands of cancer cells (Shoshan-Barmatz et al. 2010a). As such, down-regulation of VDAC1 expression results in reduced metabolite exchange between mitochondria and the cytosol, leading to mitochondrial dysfunction and arrest of cell growth, demonstrating the essential role of VDAC1 in energy production and cell growth (Abu-Hamad et al. 2006; Koren et al. 2010). Similarly, alterations of mitochondrial function are linked to VDAC1 closure, which limits the normal flow of metabolites in and out of mitochondria (Vander Heiden et al. 2000). Indeed, previous studies from our lab have demonstrated that down-regulation of VDAC1 expression by hVDAC1-shRNA disrupts energy production and cell growth and inhibits tumor development in an animal model (Abu-Hamad et al. 2006; Koren et al. 2010).

Diminished VDAC1 expression by means of shRNA strongly inhibited cell proliferation and cancer cell growth *in vitro* in cell cultures, and *in vivo* using animal models (Abu-Hamad et al. 2006; Koren et al. 2010). siRNA at nanomolar concentrations silenced VDAC1 expression in all tested cell lines, including lung cancer A549, prostate cancer PC-3, glioblastoma U87 and hepatocellular carcinoma HepG2 cells, leading to a remarkable decrease in VDAC1 protein levels and inhibition of cancer cell growth (over 90 %), which persisted up to 144 h post-transfection (Fig. 16.3A, B) (Arif et al. 2014). Cells expressing low VDAC1 levels contained low ATP levels, suggesting limited metabolite exchange between mitochondria and cytosol (Abu-Hamad et al. 2006; Koren et al. 2010).

In vivo experiments using xenograft lung cancer (Arif et al. 2014) and glioblastoma (Fig. 16.3C, D) mouse models showed that chemically modified VDAC1-siRNA not only inhibited tumor growth but also resulted in tumor regression (Arif et al. 2014). Moreover, immunohistochemical staining with anti-VDAC1 antibodies of tumor sections from scrambled- and VDAC1-siRNA-treated mice showed strong staining of untreated tumor sections as expected from cancer cells, while very weak staining was seen in the treated tumor (Fig. 16.3E) The discriminatory effects of siRNA on cancerous and non-cancerous cancer cells (Arif et al. 2014) may result from the high expression levels of VDAC1 in tumors, suggesting VDAC1 to be important for cancer cell development and survival. Thus, siRNA-VDAC1-mediated inhibition of cancer cell growth and tumor development as a result of disabling the abnormal metabolic behavior of cancer cells points to an approach to treat cancer.

Fig. 16.3 (contiuned) and human proteins. Representative sections from each group revealed strong staining in scrambled siRNA-injected tumors, with homogenous and strong staining being seen with anti-VDAC1 antibodies. The sections from the hVDAC1-siRNA -injected tumor showed non-homogenous staining, with strong staining representing a tumor containing U87 cancer cells while some non-stained areas most likely represent cells of mouse origin (*marked arrows*). *NS* represents staining with only secondary antibodies. *Bars* represent 5 μm

16.1.5 Apoptosis, VDAC1 and Cancer

The mitochondria-mediated apoptotic pathway can be triggered by diverse intracellular signals, such as oxidative stress, chemotherapeutic drugs, Ca^{2+} overload and DNA damage, and is activated when the pro-apoptotic signals overcome anti-apoptotic signals, leading to mitochondria permeabilization and release of inter-membrane (IMS) apoptogenic proteins (e.g. cytochrome *c* (Cyto *c*), AIF, Smac/DIABLO) (Shoshan-Barmatz et al. 2010b; Zaid et al. 2005). The released Cyto *c*, as a constituent of the apoptosome, activates cysteine-aspartic protease caspase-9 that in turn activates the executioner caspases, caspase-3 and -7, leading to cell destruction (Kroemer et al. 2007).

It remains unclear how apoptotic initiators that reside in the IMS cross the OMM and released into the cytosol. Several models have been proposed, such as the formation of the PTP, the assembly of a protein channel composed of Bax, Bak or both, of Bax and VDAC1, or of VDAC1 alone (Fig. 16.2B). Indeed, it is now recognized that VDAC1 acts as a key player in mitochondria-mediated apoptosis (Lemasters and Holmuhamedov 2006; Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz and Mizrachi 2012). VDAC1 participates in apoptosis via the release of mitochondrial pro-apoptotic proteins to the cytosol, and interacts with apoptosis regulatory proteins, such as Bcl-2, Bcl-xL (Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Malia and Wagner 2007; Shimizu et al. 2000; Sugiyama et al. 2002; Tsujimoto and Shimizu 2002) and HK (Abu-Hamad et al. 2009; Abu-Hamad et al. 2008; Arzoine et al. 2009; Zaid et al. 2005) (see Sect. 1.6), that are over-expressed in many cancers (Grobholz et al. 2002; Mathupala et al. 2009). The proposed VDAC1 structure mediating Cyto *c* release corresponds to a VDAC1 oligomer, forming a channel large enough to enable release of Cyto *c* and leading to apoptosis induction (see Sect. 1.5.1).

16.1.5.1 VDAC1 Oligomerization, Function, Modulation and Apoptosis Induction

Purified and membrane-embedded VDAC1 were shown to assemble into dimers, trimers, tetramers and higher order oligomers, as revealed by chemical cross-linking and fluorescence resonance energy transfer (FRET) analysis (Keinan et al. 2010; Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Golan 2010; Shoshan-Barmatz and Mizrachi 2012; Zalk et al. 2005). In addition, the NMR-based structure of recombinant hVDAC1 implied that it forms a dimer of monomers arranged in parallel (Bayrhuber et al. 2008) (Fig. 16.1Ad), while analysis of the crystal packing of mVDAC1 revealed strong anti-parallel dimers that further assemble into hexamers (Ujwal et al. 2009).

We have demonstrated that VDAC1 oligomerization is highly increased upon apoptosis induction, as revealed by chemical cross-linking, or as directly monitored in living cells using BRET (bioluminescence resonance energy transfer), and is accompanied by conformational changes in the protein (Keinan et al. 2010; Shoshan-Barmatz et al. 2008). Enhancement of VDAC1 oligomerization was obtained regardless of the cell type or apoptosis inducer used, including STS, curcumin, As_2O_3, etoposide, cisplatin, selenite,

TNF-α, H_2O_2 or UV (see also Fig. 16.6B), all capable of activating mitochondria-mediated apoptosis, yet acting through different mechanisms (Keinan et al. 2010). Moreover, VDAC1 over-expression resulted in VDAC1 oligomerization and apoptosis in the absence of any apoptotic stimulus (Shoshan-Barmatz et al. 2008; Weisthal et al. 2014). Structural and computational-based approaches, in combination with site-directed mutagenesis, cysteine replacement and chemical cross-linking, identified contact sites between VDAC1 molecules in dimers and higher order oligomers (Geula et al. 2012b). These and other findings (Abu-Hamad et al. 2009; Keinan et al. 2010; Shoshan-Barmatz et al. 2006, 2008b; Zalk et al. 2005) have led to the proposal that VDAC1 oligomers form large protein-conducting channels, offering the pathway for Cyto *c* release. This proposed mechanism is further supported by the findings that apoptosis inducers up-regulate VDAC1 expression levels, shifting monomeric to oligomeric VDAC1, leading to Cyto *c* release and subsequently, to apoptosis (see Sect. 1.8.2). The oligomerization of VDAC1 associated with apoptosis induction provides a new approach for developing a new class of drugs, directly targeting VDAC1 to induce its oligomerization.

16.1.6 Cancer Cells Avoid Apoptosis

Many malignant cells arise from the multi-step process of tumorigenesis, involving accumulation of inherited or acquired genetic alterations that protect malignant cells from apoptosis (Hanahan and Weinberg 2011). In fact, highly aggressive treatment-resistant tumors employ multiple pathways to avoid apoptosis (Hanahan and Weinberg 2011). Over-expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL is seen in numerous cancer types, with the expression of these proteins correlating with resistance to chemotherapy-induced apoptosis (Adams and Cory 2007; Llambi and Green 2011). Thus, specific induction of apoptosis in cancer cells is an effective approach for killing cancer cells. However, conventional apoptosis-inducing chemotherapy is limited by a lack of specificity, resistance, and toxicity to normal cells. Therefore, the potential benefits of developing novel, target-specific anti-cancer drugs interfering with anti-apoptotic proteins interacting with VDAC1 are enormous.

16.1.6.1 VDAC1-Interacting Proteins

VDAC1, located in the OMM, serves as an anchor protein for a diverse set of molecules that interact with the mitochondria. VDAC1 displays binding sites for glycerol kinase, HK, glyceraldehyde 3-phosphate dehydrogenase, creatine kinase, C-Raf kinase, ANT (adenine nucleotide translocase), TSPO (translocator protein), tubulin, the dynein light chain (mtHSP70), the ORDIC channel, gelsolin, actin and superoxide dismutase 1 (SOD1), as well as Bcl-2 family members (Hanahan and Weinberg 2011; Shoshan-Barmatz et al. 2006, 2010a, 2013; Shoshan-Barmatz and Mizrachi 2012). Serving as an anchor point for apoptosis-regulating proteins, such as HK and Bcl-2 family members, some of which are also highly expressed in many cancers

(Adams and Cory 2007; Grobholz et al. 2002; Llambi and Green 2011; Mathupala et al. 2009), VDAC1 can be considered as a key protein in apoptosis regulation. These interactions can be prevented by VDAC1-based peptides that bind specifically to HK (Abu-Hamad et al. 2009; Abu-Hamad et al. 2008; Arzoine et al. 2009), Bcl-2 or Bcl-xL (Abu-Hamad et al. 2009; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Malia and Wagner 2007;), abolishing the cancer cell's abilities to bypass the apoptotic pathway. Here, we focus on the interaction of VDAC1 with HK, Bcl-2 and Bcl-xL.

VDAC1-Hexokinase Interaction

Cancer cells rely on glycolysis as the main energy-generating pathway (i.e. the Warburg effect) and as a source of building blocks for proteins, nucleotides and lipids (Hanahan and Weinberg 2011; Mathupala et al. 2009). The metabolic reprogramming of cancer cells includes marked over-expression of the mitochondrial-bound HK-I and HK-II isoforms (Grobholz et al. 2002; Mathupala et al. 2009), enzymes considered as rate-limiting for glycolysis and serving as the biochemical gate of this pathway (Mathupala et al. 2006; Shoshan-Barmatz et al. 2010a).

By binding to VDAC1 (Abu-Hamad et al. 2009; Abu-Hamad et al. 2008; Arzoine et al. 2009; Zaid et al. 2005), HK provides both a metabolic benefit and apoptosis-suppressive capacity that offers the cell a proliferative advantage while increasing its resistance to chemotherapy. The VDAC1-bound HK complex facilitates and promotes the high glycolytic tumor phenotype. HK, by association with VDAC1, gains direct access to mitochondrial ATP, reaching VDAC1 via the ANT in the IMM, allowing it to phosphorylate and 'trap' any incoming glucose (Pedersen 2008). With this direct coupling of mitochondrially generated ATP to incoming glucose via VDAC1-bound HK, mitochondria regulate glycolytic flux with that of the TCA cycle and ATP synthase to balance the energy requirements of the tumor cell with the biochemical requirements for metabolites (i.e. the anaplerotic and cataplerotic pathways, respectively) or metabolic precursors that are required by the tumor (Mathupala et al. 2006; Shoshan-Barmatz et al. 2010a, b). Thus, both the glycolytic pathway and other seminal metabolic pathways, like the pentose phosphate shunt, are regulated via the energy-coupling resulting from the formation of a VDAC1-HK complex. Moreover, the up-regulation of HK expression in tumor cells (Grobholz et al. 2002; Mathupala et al. 2009) and its binding to VDAC1 provide both a metabolic benefit and apoptosis-suppressive capacity that offers the cell a growth advantage and increases its resistance to chemotherapy (Arzoine et al. 2009). As part of a system that impacts cell growth, the VDAC1-HK complex thus offers a remarkable target for cancer therapy (see below).

VDAC1 Interaction with Bcl-2 Family Proteins

One of the hallmarks of cancer cells is their resistance to apoptosis through the development of a variety of strategies, including quenching of the mitochondrial apoptotic pathway by over-expression of anti-apoptotic proteins of the Bcl-2 family that are associated with resistance of tumors to chemotherapy (Adams and Cory 2007; Llambi and Green

2011). These apoptosis regulator proteins comprise both pro- and anti-apoptotic members and are classified by sequence homology based on the presence of up to four α-helical domains, termed Bcl-2 homology (BH) 1–4 domains (Adams and Cory 2007).

The mechanisms by which Bcl-2 family proteins regulate apoptosis are still not fully understood, yet it is well established that their activities are mediated via interactions with the mitochondria and controlling permeability of the OMM to Cyto *c* and other apoptotic factors (Adams and Cory 2007; Llambi and Green 2011). Moreover, accumulated evidence suggests that Bcl-2 family members act via interaction with VDAC1 (Abu-Hamad et al. 2009; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Malia and Wagner 2007; Shimizu et al. 2000; Sugiyama et al. 2002; Tsujimoto and Shimizu 2002; Yamagata et al. 2009). VDAC1 has been shown to interact with the Bax/Bak complex, Bcl-2 and Bcl-xL, as well as with Bax and Bim. Bax was found to increase VDAC1 conductance in one (Banerjee and Ghosh 2004), although not in a second study (Rostovtseva et al. 2004). The involvement of VDAC1 in Bax-mediated apoptosis has been proposed based on findings that in VDAC1-depleted cells, cisplatin-induced conformational activation of Bax was inhibited (Tajeddine et al. 2008) and because anti-VDAC antibodies inhibited Bax- and Bim-induced release of Cyto *c* (Shimizu et al. 2001). Moreover, it was shown that Bcl-xL can modify the VDAC1 oligomeric state by shifting the equilibrium from a monomeric to a dimeric state (Malia and Wagner 2007). Taken together, these data indicate that both the pro- and anti-apoptotic activities of Bcl-2 family proteins are mediated via interactions with VDAC1. Hence, interfering with these interactions should facilitate apoptosis induction and enhance the therapeutic effects of chemotherapeutic agents, like cisplatin. Such an approach involving VDAC1-based peptides interacting with Bcl-2 (Arbel and Shoshan-Barmatz 2010) and Bcl-xL (Arbel et al. 2012) has been applied to prevent the anti-apoptotic activities of these proteins, as considered below (Sect. 1.7).

16.1.6.2 HK, Bcl-2 and Bcl-xL Interact with the VDAC1 N-Terminal Domain

The N-terminal domain of the VDAC1 protein was shown to be accessible to anti-VDAC1 antibodies raised against this domain (Abu-Hamad et al. 2006). The same domain is exposed to kinases, as threonine-13 undergoes phosphorylation (Distler et al. 2007). Furthermore, the N-terminal domain of VDAC1 acts as a recruiting site for HK1, Bcl-2 and Bcl-xL and thus is a key structural feature mediating VDAC1 interaction with anti-apoptotic proteins and enabling their function (Abu-Hamad et al. 2009; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Arzoine et al. 2009; Malia and Wagner 2007; Shimizu et al. 2000; Sugiyama et al. 2002; Tsujimoto and Shimizu 2002), suggesting the exposure of this VDAC1 region outside the pore. Indeed, it has been further demonstrated the N-terminal region of VDAC1 is loosely attached to the barrel wall and can undergo translocation out of the pore (Geula et al. 2012a). Such movement of the N-terminal domain allows VDAC1 to interact with the anti-apoptotic proteins, HK, Bcl-xL and Bcl-2 (Abu-Hamad et al. 2009; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010). Thus, a peptide corresponding to the VDAC1-N-terminal domain can be used as a decoy

to compete with native VDAC1 for interactions with HK, Bcl-2 and Bcl-xL, thereby preventing their anti-apoptotic activities.

16.1.7 VDAC1-Based Peptides as a Potential Anti-cancer Agent

The analysis of therapeutic targets and drug resistance-conferring gene mutations in cancers opens new perspectives in cancer therapy. However, the heterogeneity and complexity of malignant tumors have changed how we think about the initiation, progression, diagnosis, and management of cancer, thus requiring multiple targets or the use of a pan-drug. Although small molecule drugs remain standard treatment for the disease, the search for new anti-cancer agents is mostly driven by the desire to identify compounds which do not possess the excessive toxicity of currently available chemotherapeutic drugs and that can overcome the resistance that eventually emerges towards those drugs. As such, new therapeutic strategies addressing such requirements are based on engineered genes, antibodies, proteins, peptides and siRNA, collectively known as bio-therapeutics.

Therapeutic peptides represent an important class of anti-cancer agents, owing to their stability, lack of immunogenicity, ability to penetrate tumor tissue, and relatively low cost of synthesis (Raucher et al. 2009). The notable expansion of peptide therapeutics development and increased marketing over the last few years could be a harbinger for innovative peptide-based drugs (Kaspar and Reichert 2013). Accordingly, recent advances in peptide pharmacokinetics (Kaspar and Reichert 2013) now allow for overcoming most of the limitations associated with the use of peptides as therapeutic agents. For instance, peptide stability can be increased by introducing unnatural amino acids during organic synthesis (for example using D-amino acids instead of L-amino acids) or by attaching the peptide to a macromolecular carrier. Such innovations have revitalized the use of peptides as therapeutic agents, with six new peptides having been approved for marketing in the US in 2012, including carfilzomib for the treatment of multiple myeloma. Today, many other candidates are in the pipeline, including 74 peptides in Phase I/II, Phase II or Phase III studies (Kaspar and Reichert 2013).

Several peptides have been successfully tested for their abilities to disrupt various protein-protein interactions in metabolically aberrant cancer cells, and consequently induce apoptosis. Synthetic peptides mimicking the BH3 domain of pro-apoptotic Bcl-2 proteins have been shown to activate mitochondrial apoptosis in proliferating cancer cells (Letai et al. 2002). Also, cell-permeable second mitochondria-derived activator of caspases (SMAC)-based peptides able to inhibit inhibitors of apoptosis (IAPs), were shown to have an anti-tumor effect in glioma cells (Fulda et al. 2002) and enhance the apoptotic effect of chemotherapeutic agents both *in vivo* and *in vitro* (Arnt et al. 2002). Moreover, HK-based peptides were also shown to selectively induce apoptosis by detaching mitochondria-bound HK (Chiara et al. 2008).

Relying on point mutations, we have identified VDAC1 domains and amino acid residues important for interactions with HK, Bcl-2 and Bcl-xL, and designed VDAC1-based peptides targeting these interactions (Abu-Hamad et al. 2009; Abu-

Hamad et al. 2008; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Arzoine et al. 2009;). These peptides are designed to serve as "decoy" peptides that compete with VDAC1 for the Bcl2-, Bcl-xL- and HK-VDAC1 interaction sites and consequently interrupt their anti-apoptotic activities. As the targets of VDAC1-based peptide are intracellular proteins, several cell-penetrating versions of the peptides were designed and tested (Fig. 16.4A, D). These peptides were shown to promote cell death in a panel of genetically characterized cell lines derived from different human cancers (Prezma et al. 2013; Zooravlev et al.).

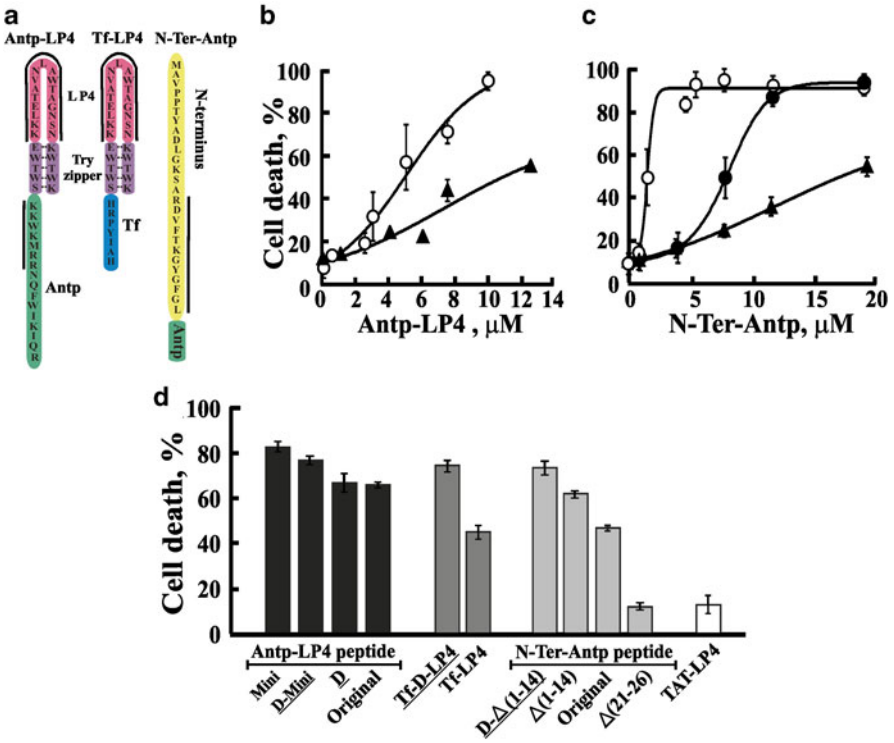

Fig. 16.4 VDAC1-based peptides induce cell death. (**a**) Schematic illustration of the structures of VDAC1-based peptides. Loop-shaped Antp-LP4 and Tf-LP4 and N-Ter-Antp VDAC1-based peptides are shown. VDAC1-derived sequences, LP4 and N terminus are in *pink* and *yellow*, respectively. The cell-penetrating peptide (Antp) is in *green*, and the loop shape stabilized by a tryptophan zipper is in *purple*. The *solid black lines* mark the amino acids most important for peptide. (**b**, **c**) Antp-LP4 and N-Ter-Antp peptides effectively induced cell death of various cancer lines but to a lesser extent in non-cancerous T-Rex cells. Two cancer cell lines, PC3 (**o**, prostate adenocarcinoma, n = 3) and A549 (●, alveolar adenocarcinoma, n = 3), and immortalized T-Rex (▲, embryonic kidney fibroblasts, n = 3) cells were incubated for 6 h in a serum-free-medium containing the indicated concentrations of Antp-LP4 (**b**) or N-Ter-Antp (**C**) peptide. Cells stained with PI reflecting cell death were analyzed by flow cytometry. (**d**) Cell death induction by various VDAC1-based peptides. MEC-1 cells were incubated for 1.5 h with 5 μM of the indicated peptides and the percent of cell death as analyzed by PI staining is presented

Antp-LP4 peptide is a loop-shaped cell-penetrating peptide comprising Antp (Penetrating), a 16 residue sequence from the *Drosophila* antennapedia-homeodomain, fused to a VDAC1-derived sequence (designated LP4). The SWTWE sequence at the N-terminal and the KWTWK sequence at the C-terminal ends of the VDAC1-derived peptide allow for formation of a tryptophan zipper and a stable β-hairpin (Cochran et al. 2001), mimicking the LP4 loop in the native VDAC1 protein (Fig. 16.4A). The VDAC1-based Antp-LP4 peptide diminished the anti-apoptotic effects of HK-I, Bcl-2 or Bcl-xL (Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Arzoine et al. 2009) and induced cell death in several cancer cell lines, while being less effective in non-cancerous cells (Prezma et al. 2013) (Fig. 16.4B, C). Shortening the Antp moiety by eight residues yielded a minimal sequence conferring cell permeability resulted in a highly active peptide (Min-Antp-LP4) (Prezma et al. 2013). Furthermore, using D-enantiomers to generate an all-D amino acid-containing version of Antp-LP4 generated a peptide that was as, if not more effective than the corresponding L-version in inducing cell death (Fig. 16.4D) (Prezma et al. 2013).

Tf-LP4 As human transferrin receptor (hTfR) is over-expressed in many cancers (Daniels et al. 2012), the cell-penetrating Antp sequence of Antp-LP4 was replaced with a hTfR-recognition sequence, HAIYPRH (Daniels et al. 2012), to form Tf-LP4 (Fig. 16.4A). Whereas all D-Tf-LP4 was less active, the peptide was as effective as the all L-version when only the LP4 portion comprised D-amino acids (Tf-D-LP4) (Fig. 16.4D).

N-Ter-Antp This sequence was selected based on the findings that N-terminal domain-truncated VDAC1 had lost the ability to bind HK, Bcl-2 or Bcl-xL (Abu-Hamad et al. 2009; Abu-Hamad et al. 2008; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Arzoine et al. 2009; Zaid et al. 2005). A cell-penetrating VDAC1-derived peptide, (1-26)-N-terminal peptide (N-Ter-Antp) (Fig. 16.4A) interacted with HK, Bcl-2 and Bcl-xL and inhibited the anti-apoptotic effects of these proteins (Abu-Hamad et al. 2009; Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010; Arzoine et al. 2009;). The N-Ter-Antp peptide induced cell death only when Antp was C-terminally fused (Fig. 16.4B) Shortening the (1-26)-N-Ter-Antp peptide by up to 14 residues [Δ(1-14)N-Ter-Antp] yielded an active peptide, while deleting six residues from the C-terminus of the peptide, including the GxxxG motif, yielded a non-active peptide (Fig. 16.4D). The D-enantiomer of N-Ter-Antp was two-fold more active than was the L-peptide (Fig. 16.4D) while fusing N-Ter-Antp with Tf yielded a less active peptide.

TAT-LP4 This peptide, bearing the HIV-1 Tat-dependent transactivation peptide TAT shown to confer cell permeability (Brooks et al. 2005), was less effective in inducing cell death (Fig. 16.4D) and thus offered no advantage over Antp-LP4 or Tf-LP4.

Following the design of over 27 versions of cell-penetrating VDAC1-based peptides, involving peptide cyclization, deletion of amino acids, blocking the peptide N-terminus by acetylation or the C-terminus by amidation and using D-enantiomers, strategies aimed at shortening the peptides and preventing their degradation by peptidases, four optimized peptides, Tf-D-LP4, D-Antp-LP4, D-MinAntp-LP4 and D-Δ(1-14)-N-Ter-Antp (Fig. 16.4D undelined), were selected for use in *in vivo* experiments.

16.1.8 The Mode of Action of VDAC1-Based Peptides

The proposed mode of action of the VDAC1-based peptides in cell death induction (Fig. 16.6) is based on the findings that the peptides have a triple action: (i) Impairing cell metabolism and energy homeostasis; (ii) preventing the anti-apoptotic activities of Bcl-2, Bcl-xL and HK and (iii) inducing apoptosis (Abu-Hamad et al. 2009; Arzoine et al. 2009; Prezma et al. 2013).

16.1.8.1 VDAC1-Based Peptides Energy and Metabolism Impairment

Altered energy metabolism, including enhanced aerobic glycolysis, is a fundamental phenotype of malignant tumors (Ferreira 2010; Hanahan and Weinberg 2011; Kaelin and Thompson 2010). Mitochondrial-bound HK is markedly elevated in highly glycolytic cancer cells (Mathupala et al. 2009), supporting aerobic glycolysis (Mathupala et al. 2006) critical for the stability of mitochondria (Wenner 2010) and conferring resistance to apoptosis (Shoshan-Barmatz et al. 2010a; Shulga et al. 2009). Over-expressed VDAC1 in cancers (Grills et al. 2011; Lan et al. 2010; Prezma et al. 2013) presents increased numbers of anchoring sites for HK, allowing direct excess to mitochondrial ATP and an increased glycolytic rate (see Sect. 1.4) (Shoshan-Barmatz et al. 2010a). This coupling is impaired by HK-detaching peptides, leading to decreased glycolysis, energy and precursor production, allowing apoptosis induction (Abu-Hamad et al. 2009; Arzoine et al. 2009). Indeed, we have previously demonstrated that Antp-LP4 detached HK from mitochondrial VDAC1 (Arzoine et al. 2009; Prezma et al. 2013) (Fig. 16.5B) VDAC1-based peptide-induced HK displacement affects overall cellular bioenergetics, as reflected in the peptide-induced decrease in $\Delta\Psi m$ and cellular ATP levels (Arzoine et al. 2009; Prezma et al. 2013).

16.1.8.2 VDAC1-Based Peptides Prevent the Anti-apoptotic Activities of Anti-apoptotic Proteins

Over-expression of anti-apoptotic proteins is a known anti-defense mechanism of cancer cells (Adams and Cory 2007; Grobholz et al. 2002; Llambi and Green 2011). VDAC1, by providing binding sites for Bcl-2 and Bcl-xL, allows these proteins to mediate their anti-apoptotic effects (Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010). Bcl-2 and Bcl-xL directly interact with VDAC1. VDAC1-based peptides representing the N-terminal domain and an internal sequence (LP4) interacted with both proteins to prevent their anti-apoptotic activities (Arbel et al. 2012; Arbel and Shoshan-Barmatz 2010). These findings suggest that interfering with Bcl-xL binding to the mitochondria by VDAC1-based peptides may serve to induce apoptosis in cancer cells and potentiate the efficacy of conventional chemotherapeutic agents.

16.1.8.3 VDAC1-Based Peptides Induce Apoptosis

The proposed mechanism for VDAC1-based peptide-mediated induction of apoptosis is related to the over-expression of VDAC1 and its oligomerization, leading to the release of Cyto *c* and subsequent apoptosis (Prezma et al. 2013). We have previously shown that VDAC1 oligomerization is coupled to apoptosis induction (Keinan et al. 2010; Shoshan-Barmatz and Mizrachi 2012). As VDAC1 is over-expressed in many cancer cells, displacement of HK, Bcl-xL and Bcl-2 from VDAC1 encourages the high concentration of free VDAC1 molecules to oligomerize, leading to Cyto *c* release and apoptosis (Fig. 16.5B, C). As such, Antp-LP4 induced release of Cyto *c* and other molecular hallmarks of apoptosis, as revealed by both confocal and electron microscopy, including membrane blebbing, phosphatidylserine surface exposure, and nuclear condensation and fragmentation (Prezma et al. 2013) (Fig. 16.5C). This multiple mode of action may explain the high effectiveness of the cell-penetrating VDAC1-based peptides with perceived specificity toward cancerous cells.

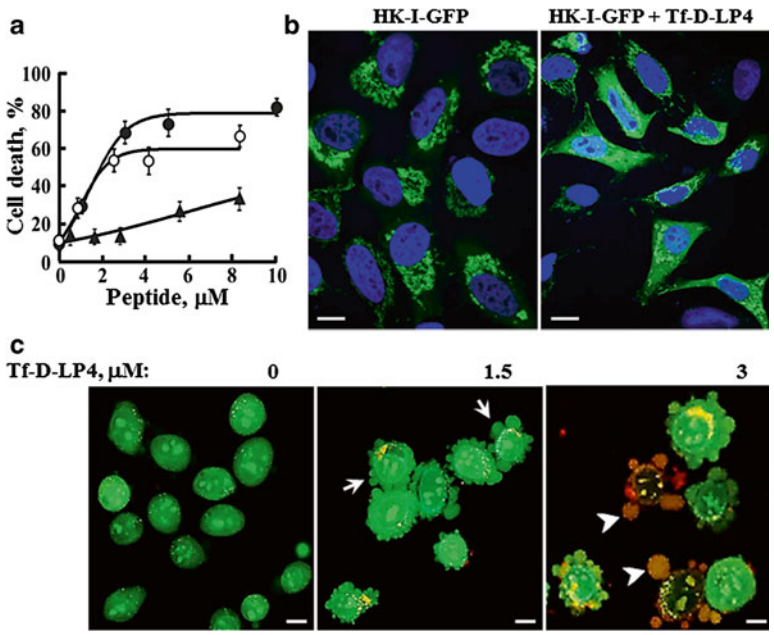

Fig. 16.5 VDAC1-based peptides act by detaching mitochondria-bound HK and inducing apoptosis. (**A**) Cell death induction by VDAC1-based peptides. MEC-1 cells were incubated for 90 min with various concentrations of Tf-D-LP4 (●), Tf-LP4 (**o**) or D-Tf-LP4 (▲) peptides and the cell death was analyzed by PI staining. (**B**) Detachment of mitochondria-bound HK-I-GFP by VDAC1-based peptide. HeLa cells were transfected to express HK-I-GFP. After 24 h cells were incubated without (*left)* or with Tf-D-LP4 peptide (2 μM, 3 h) and visualized by confocal microscope. *Scale bars*, 10 μm (**C**) VDAC1-based peptide induces apoptosis. HeLa cells were treated with the indicated concentrations of TF-D-LP4 for 2.5 h and then stained with acridine orange/ethidium bromide. The *arrow* and *arrowhead* indicate cells in the early (membrane blebbing) and late apoptotic (fragmented nuclei), stages, respectively. *Scale bars*, 5 μm

To conclude, VDAC1-based peptides act very rapidly (within hours) and very effectively at low micromolar concentrations to induce cell death in a variety of cell lines, regardless of their cancer origin or their genetic makeup. As such, VDAC1-based peptides can be considered as pan-drugs. With a wide therapeutic index, these peptides represent a good drug candidate for treating many cancers. This is very important in view of heterogeneity, metastatic formation and modifications acquired during tumor development.

16.1.9 Small Molecules Acting via Direct Interactions with VDAC1 or by Inducing VDAC1 Over-Expression

VDAC1 is over-expressed in a diverse set of cancer types (Grills et al. 2011; Lan et al. 2010; Prezma et al. 2013). This, together with its major role in mitochondrial-mediated apoptosis (see Sect. 1.5), makes VDAC1 a potent target for newly developed anti-cancer drugs (Granville and Gottlieb 2003; Shoshan-Barmatz et al. 2010a). Studies demonstrating that some drugs mediate their effects via VDAC1 are presented below.

16.1.9.1 Apoptosis-Agents Directly Interact with and Modify VDAC1 Activity

G3139 (oblimersen), an 18-mer phosphorothioate anti-sense oligonucleotide targeted to the initiation codon region of Bcl-2 mRNA, directly binds and reduces the channel conductance of bilayer-reconstituted VDAC (Lai et al. 2006). G3139 induced caspase-dependent apoptosis via the intrinsic Bcl-2-independent pathway (Lai et al. 2005; Lai et al. 2006).

Avicins represent a novel class of plant stress metabolites that exhibit cytotoxic activity in tumor cells, as well as anti-inflammatory and anti-oxidant properties capable of perturbing mitochondrial function and initiating apoptosis in tumor cells. Avicins interact with bilayer-reconstituted VDAC1 to reduce its channel conductance (Haridas et al. 2007) and permeabilize the OMM to induce Cyto *c* release (Lemeshko et al. 2006).

Fluoxetine (Prozac), a clinically-used anti-depressant compound which acts on multiple transporters and channels, enhanced cell proliferation and prevented (Lee et al. 2001) or enhanced apoptosis (Serafeim et al. 2003) in various cell lines. Fluoxetine was shown to (a) interact directly with purified bilayer-reconstituted VDAC and decrease its channel conductance, (b) prevent the opening of the PTP, and (c) inhibit the release of Cyto *c* and apoptotic cell death induced by STS (Nahon et al. 2005; Thinnes 2005). Thus, fluoxetine may mediate its effects on apoptosis by interacting with VDAC1.

Cisplatin is a widely used anti-cancer drug that acts by inducing apoptosis via forming inter- and intra-strand nuclear DNA cross-links. Mitochondria have also been implicated as a cisplatin target (Cullen et al. 2007; Yang et al. 2006). Cisplatin binds to both mitochondrial DNA and VDAC1 (Yang et al. 2006) and modulates

VDAC1 activity (Castagna et al. 2004). Additionally, silencing VDAC1 expression inhibited cisplatin activation of Bax and apoptosis (Tajeddine et al. 2008). These findings suggest that VDAC1 may serve as a cisplatin receptor in apoptotic pathways (Thinnes 2009).

Acrolein (2-propen-1-al), the most reactive of the α,β-unsaturated aldehydes and a toxic compound, was proposed to react with DNA and proteins. VDAC was recently identified as a selectively oxidized target in Alzhimer's disease (AD) brain tissue, being significantly carbonylated by acrolein (Sultana et al. 2006).

Endostatin (ES) has been shown to promote PTP opening via VDAC. Silencing VDAC1 expression by siRNA attenuated ES-induced apoptosis in endothelial cells (Yuan et al. 2008).

Methyl jasmonate (MJ) is a natural cyclopentanone lipid belonging to the jasmonate (JA) family of plant oxylipin stress hormones (oxygenated fatty acids) (Raviv et al. 2013). MJ directly interacts with VDAC1 and reduces its channel conductance (Goldin et al. 2008). MJ also detaches HK from VDAC1 to abrogate the anti-apoptotic activity of the VDAC1-HK complex. This leads to a dissociation of glycolytic and mitochondrial metabolic functions, as well as decreasing the mitochondrial membrane potential ($\Delta\psi m$), favoring Cyto *c* release. This causes ATP depletion, activates pro-apoptotic proteins and inactivates anti-apoptotic proteins (Goldin et al. 2008). It should be noted that MJ-induced cytotoxicity may, however, result from its other observed effects (Raviv et al. 2013).

16.1.9.2 Pro-apoptotic-Agents Up-Regulating VDAC1 Expression Level

Several studies demonstrated that apoptosis-inducing agents and treatments resulted in up-regulation of VDAC1 expression. Prednisolone treatment of acute lymphoblastic leukemia cell lines resulted in enhanced VDAC1 protein levels (Jiang et al. 2011). Cisplatin induced VDAC1 over-expression in reagent-sensitive but not in resistant cervix squamous carcinoma cell lines (Castagna et al. 2004). It was also shown that over-expression of VDAC1 sensitized carcinoma cells to apoptosis induced by cisplatin, mechlorethamine, and its derivative, melphalan (Sharaf el dein et al. 2012). Arbutin (hydroquinone-O-beta-D-glucopyranoside), a tyrosinase inhibitor and a potential anti-cancer agent, induced apoptosis by causing VDAC1 over-expression (Cheng et al. 2007; Nawarak et al. 2009). Somatostatin, used in treatment of advanced prostate cancer, was found to up-regulate the expression of VDAC1 and VDAC2 in the LNCaP prostate cancer cell line (Liu et al. 2007). In addition, both UV irradiation and ROS were shown to up-regulate VDAC1 expression (Jung et al. 2007; Voehringer et al. 2000).

Indeed, over-expression of VDAC1 was obtained upon cell treatment with cisplatine, H_2O_2, As_2O_3 or etoposide, which in turn up-regulated VDAC1 expression, leading to VDAC1 oligomerization (Weisthal et al. 2014) (Fig. 16.6A, B). Hence, while accepted modes of actions have been described for the majority of apoptosis inducers, the results presented above propose an additional mode of action for apoptosis stimulus involving up-regulation of VDAC1 expression is proposed (Weisthal

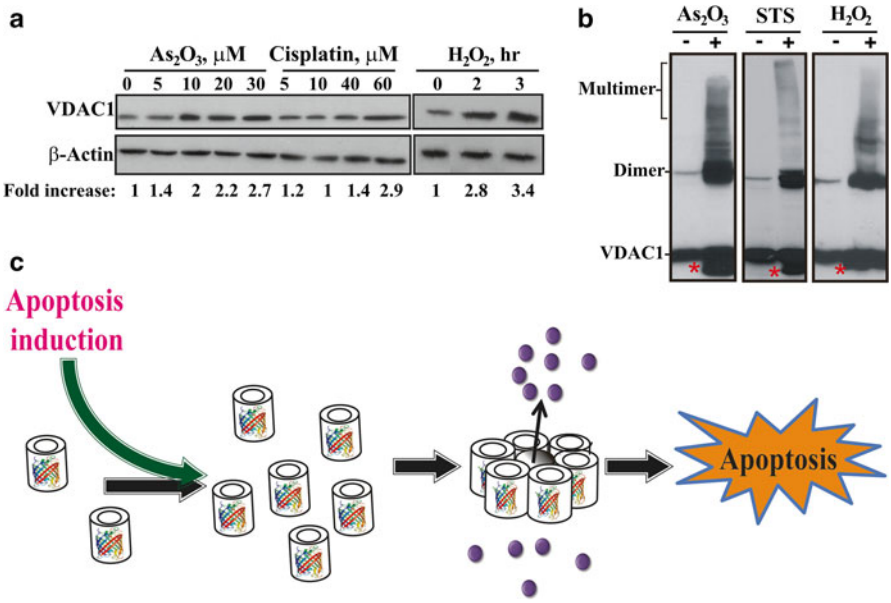

Fig. 16.6 Apoptosis inducers enhance VDAC1 oligomerization and expression. (**A**) HeLa cells were incubated with the indicated concentrations of As_2O_3 (5–30 μM, 16 h), cisplatin (5–60 μM, 16 h), or H_2O_2 (0.8 mM, 2–3 h). VDAC1 expression levels were analyzed by immunoblotting followed by quantitative analysis of VDAC1 expression, presented at the bottom of each blot as fold-increase. (**B**) HeLa cells were incubated with As_2O_3 (20 μM, 16 h), STS (1 μM, 4 h), or H_2O_2 (0.8 mM, 6 h), and VDAC1 oligomerization was revealed using EGS-based cross-linking followed by SDS-PAGE and immunoblotting using anti-VDAC1 antibodies. The positions of VDAC1 monomers to multimers are indicated and the *red asterisks* indicate an anti-VDAC1 antibody-labeled protein band migrating below the position of monomeric VDAC1. (**C**) Proposed model suggesting that apoptosis induction by a given stimulus causes enhanced VDAC1 expression that in turn shifts the equilibrium towards the VDAC1 oligomeric states, allowing Cyto *c* (*purple circles*) release from the inner mitochondria space, leading to apoptotic cell death

et al. 2014) (Fig. 16.6C). It was further argue that apoptosis-inducing agents act by increasing $[Ca^{2+}]i$ and that this in turn leads to an up-regulation of VDAC1 expression, followed by VDAC1 oligomerization, Cyto c release and, finally, cell death (Weisthal et al. 2014).

Importantly, the causal relationship between VDAC1 levels and drug sensitivity was emphasized in several studies. Prostate cancer cell lines that were relatively resistant to apoptosis induction by oblimersen sodium (G3139) were found to express lower levels of VDAC1 than did G3139-sensitive prostate cancer cells (Lai et al. 2006). The anti-cancer activity of furanonaphthoquinones was increased upon VDAC1 over-expression and decreased upon silencing of VDAC1 expression (Simamura et al. 2008). Thus, VDAC1 over-expression following apoptosis induction by various agents, as well as the correlation between drug efficacy and VDAC1 expression levels, suggests that the activity of numerous anti-cancer drugs and treatments is mediated via regulating VDAC1 expression levels. The findings that human

cancer cell lines express higher VDAC1 levels than do normal cells predicates that small molecules directed to VDAC1 and that enhance VDAC1 expression levels have great potential as anti-cancer drugs.

In summary, VDAC1 functions are indispensable for proper mitochondrial function, and consequently, for cell activity. This makes VDAC1 crucial for a range of cellular processes, including ATP rationing, Ca^{2+} homeostasis and apoptosis execution. These activities are regulated via interactions of VDAC1 with many proteins that are critically involved in the regulation of cell survival and cellular death pathways. Thus, using VDAC1-based agents as a novel anti-cancer approach is highly promising and is expected to be highly effective even against drug-resistant tumors. Furthermore, such treatment may enhance the sensitivity of cancer cells towards chemotherapies, thus reducing undesired side effects.

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Chapter 17
Inflammatory Dysregulation and Cancer: From Molecular Mechanisms to Therapeutic Opportunities

Colin W. Steele, Nigel B. Jamieson, and C. Ross Carter

Abstract The association between inflammation and cancer has been established for well over 100 years. Only now are we beginning to unravel the complexities of the inflammatory mechanisms that are integral to the initiation and progression of cancer. From large observational studies, to in-depth mechanistic in vivo modelling studies every aspect of inflammatory dysregulation is being examined. Better understanding of the cellular and molecular processes mediating cancer associated inflammation and the vital role it plays in cancer progression have begun to be exploited for therapeutic benefit. Here we describe the rationale for study of inflammation in cancer, provide an overview of our current understanding of inflammatory dysregulation in cancer, and assess the possible therapeutic avenues to be explored. We use pancreatic cancer, one of the most biologically diverse and aggressive examples to inform our discussion.

Keywords Inflammation • Cancer • Pancreatic • Therapy • Hallmarks of cancer • Chronic inflammation • Systemic inflammation • Tumourigenesis • COX • Cytokines • Tumour immunosurveillance • Tumour-associated macrophages • Metastasis

C.W. Steele
Beatson Institute for Cancer Research, Glasgow, UK

Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK

N.B. Jamieson
Academic Department of Surgery, College of Medical, Veterinary, and Life Sciences, University of Glasgow, Glasgow, UK

Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK

C.R. Carter (✉)
Department of Pancreaticobiliary Surgery, Glasgow Royal Infirmary, Glasgow, UK
e-mail: rosscarterno1@gmail.com

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17.1 Introduction

Cancer incidence continues to increase globally. Treatment has improved through better understanding of genetic and molecular drivers of cancer. However, in a large proportion of cancers despite treatment, disease recurrence and progression is commonplace. In diseases such as lung and pancreatic cancer significant advances in biological understanding are required in order to improve outcome for patients.

Inflammation has long been considered a tumour promoter. Indeed, Virchow was the first to associate chronic inflammation and cancer in 1863 (Balkwill and Mantovani 2012). He noted leucocyte infiltration within tumours and linked this to cancer causation in the setting of chronic inflammation. Although chronic inflammation driven cell proliferation is insufficient to drive neoplasia, persistent cell proliferation within an environment sustained by growth factors, survival signals and inflammatory cells potentiates cancer (Coussens et al. 2013). Two distinct pathways link inflammation and cancer: The first of these is extrinsically generated inflammation that predisposes to cancer formation; while the second follows tumour initiation and is intrinsically linked to the immune response to the presence of tumour cells, and in turn the harnessing of the immune system by oncogenically transformed cells in order to evade immune destruction. Inflammation generated by these processes is critical to tumour initiation, avoidance of tumour immunosurveillance, and tumour progression.

Our understanding of the complex interactions between tumours and their microenvironments have only over the past decade begun to reach a stage where the links between cancer and inflammation may be considered in terms of therapy. Hanahan and Weinberg in their 'Hallmarks of Cancer: The Next Generation' piece article identify tumour-promoting inflammation and evasion of the immune system as 'emerging hallmarks' (Hanahan and Weinberg 2011). In this chapter we will consider the rationale for targeting inflammation in cancer. We will describe current understanding of tumour promoting inflammation and ways in which tumour-microenvironment interactions permit immune cell invasion. Finally we will consider whether these processes may be reversed or ameliorated therapeutically, and use pancreatic cancer, perhaps the most aggressive and therapeutically challenging epithelial cancer, as an example of potential therapeutic strategies.

17.2 Inflammation and Cancer

17.2.1 Normal Inflammation

To understand how inflammation pertains to cancer causation we must first consider the normal physiological process of inflammation: The body's physiological response to injury and foreign antigens. Calor (heat), rubor (redness), tumor (swelling), dolor (pain) define the clinical features of inflammation and represent a homeostatic mechanism permitting host defence, tissue repair and remodelling.

Innate immune cells are the first line of defence following tissue damage. In tumours however, proliferating tumour cells evade destruction mechanisms and use secreted growth and proliferation factors from inflammatory cells to invade. Host immune defences separate into two distinct but linked pathways, the innate and adaptive immune responses. Following injury, a network of chemical signals released by damaged tissue and in particular immune cells resident in tissues propagates the immune response. Resident macrophages, dendritic cells and histiocytes recognise antigens via pattern recognition receptors. Activation results in release of inflammatory mediators including bradykinin, chemokines and cytokines such as interleukin (IL)-1, IL-6, and tissue necrosis factor alpha (TNFα). These mediators result in pain, increased vascular permeability, and immune cell migration. Innate immune cells, first neutrophils followed by monocytes (that differentiate into macrophages within tissues), are stimulated to migrate to sites of injury. Chemokines are responsible for neutrophil and macrophage activation, with resultant upregulation of adhesive integrins on the surface of these cells. L, E and P selectins are then able to recognise oligosaccharides on the surface of neutrophils and macrophages and permit adhesion via α4β1 and α4β7 integrins to vascular cell-adhesion molecule-1 (VCAM-1) and MadCAM-1 respectively (Coussens and Werb 2002). Finally these cells use diapedesis to extravasate and transmigrate to sites of injury. Production of matrix metalloproteinases (MMPs) among other extracellular proteases permits migration. Neutrophil chemotaxis is also induced via complement factor 5, leukotriene B4, kallikrein, and chemokines released by platelets (Coussens et al. 2013). Within damaged tissue, phagocytes engulf damaged cells and pathogens in addition to releasing proteolytic factors that remodel the microenvironment.

The innate ability of both neutrophils and macrophages is enabled by pattern recognition receptors including Toll-like receptors (TLRs) and various scavenger receptors. These receptors permit macrophages to activate transcription factors such as NFκB to modulate the immune response. TLR ligation also promotes phagocytosis of damaged tissue and production of reactive oxygen species in addition to other cytokines including TNFα and IL-1β. Production of these cytokines facilitates further leukocyte adherence to vasculature endothelium and promotion of inflammation at the site of injury.

Two key pro-inflammatory cytokines are TNF-α and transforming growth factor beta (TGF-β), the later of which can both positively and negatively regulate inflammation. Close regulation of these soluble inflammatory mediators maintains homeostasis and permit resolution of inflammation. The net result of inflammation is restoration of normal physiology via clearance of foreign material and healing of damaged tissue.

17.2.2 Risk Factor Exposure Links Inflammation and Cancer

Numerous established risk factors for cancer, including obesity, infectious agents, diet, and smoking directly induce inflammation (Hursting et al. 2012; Houghton 2013). Although these risk factors are known to directly influence cancer ‘initiation’ in different contexts, they are also potential tumour promoters by recruiting

pro-tumourigenic immune cells to 'initiated' tissues (Wei et al. 2010). Obesity predisposes to development of subclinical levels of inflammation particularly in visceral white adipose tissue. This inflammation is mediated by cytokines including Interleukin (IL)-6 and tissue necrosis factor (TNF)-α, and transcription factors such as NFκB and STAT3. These commonly secreted inflammatory mediators are directly involved in tumourigenesis across a number of tumour types (Fukuda et al. 2011; Ling 2012). When sustained over extended periods, this 'chronic inflammation' can provide the source of tumourigenesis in a number of different tissues (Coussens and Werb 2002). Chronic inflammatory diseases are closely associated with cancer causation. Indeed around 25 % of tumours are thought to directly arise from chronic inflammatory conditions such as: hepatitis, in hepatocellular carcinoma; or ulcerative colitis in colorectal cancer (Balkwill and Mantovani 2012). Interestingly, cellular senescence, growth arrest of cells, which occurs both during aging and following oncogene transformation of cells has traditionally been thought of as a barrier to cancer. However, contrastingly senescent cells secrete inflammatory mediators, referred to as the senescence associated secretory phenotype or SASP (Kuilman et al. 2008). Recent publications suggest that in-fact under the correct conditions senescence generated inflammation is tumour promoting and may help relate aging to increasing cancer incidence (Freund et al. 2010; Pribluda et al. 2013). In the context of chronic inflammation, it is the failure of resolution to a state of homeostasis that potentiates cancer initiation and progression.

17.3 Therapeutic Opportunities

17.3.1 Rationale for Anti-inflammatory Therapy in Cancer

17.3.1.1 Observational Studies Highlighting the Potential Role of Non-steroidal Anti-inflammatory Drugs in Cancer Prevention

Through analysis of previous randomised controlled trial data Rothwell et al. have associated daily use of aspirin with improvements in long term cancer-specific survival, short-term reductions in cancer incidence and mortality, and indeed reduction in metastases in cancers that do establish (Rothwell et al. 2011, 2012a, b). This extensive work incorporating vast numbers of cancer patients highlights the importance of inflammation in the generation of cancer across a number of different tumour types. This work also highlights that general reduction in inflammation can have a positive influence on outcome in cancer regardless of pathway dysregulation mechanism within individual cancer types. Importantly aspirin therapy had to be instituted for 5 years to show any appreciable effect on cancer mortality, with the greatest effect observed in adenocarcinoma (Rothwell et al. 2011). Furthermore, 3 years of daily aspirin therapy had to be instituted to improve cancer incidence significantly (Rothwell et al. 2012a). Although NSAIDS appear to provide an excellent preventative measure this data brings into question the role of generic

anti-inflammatories as part of a treatment regimen. The effects seen on metastasis were also most striking in patients with adenocarcinoma and underscore the critical role played by inflammation in the metastatic process. It has been inferred from these results that NSAIDs are having this effect through their effect on COX, particularly COX-2 given its overexpression in a number of solid cancers (Wang et al. 2011; Khan et al. 2011). However, there is evidence that COX independent mechanisms of NSAIDs may play a significant role in this process given the potential of NSAIDs to decrease tumour cell proliferation and influence apoptosis in vitro, independent of COX activity (Ruschoff et al. 1998; McIlhatton et al. 2007). Sulindac, a potent NSAID, can be metabolised to a COX inhibiting sulphide metabolite or a sulfone metabolite with no COX activity. Sulindac sulfone despite toxicity was able to regress familial and sporadic colonic adenoma formation in clinical trial (Arber et al. 2006; Stoner et al. 1999). Certain authors have proposed that NSAIDs may target a myriad of targets to induce apoptosis, including cyclic guanosine monophosphate phosphodiesterases, NFκB signalling, the Phosphatydinyl inositol 3 kinase (PI3K) signalling pathway among others (Gurpinar et al. 2014).

One interesting observation is the direct implication of chronic inflammation in adenocarcinoma development. Barrett's oesophagus, columnar metaplasia of the lower oesophagus, the precursor of adenocarcinoma of the oesophagus, is caused by chronic acid exposure and inflammation. Counterintuitive to traditional thinking, aspirin, is now being employed in combination with proton pump inhibitors in this high risk group to assess the effect of specifically reducing the chronic inflammatory process leading to malignancy as part of the AspECT trial (http://www.clinicaltrials.gov/show/NCT00357682. 2013).

There have been a number of preclinical trials that have assessed the effects of NSAIDs as a cancer therapeutic. In pancreatic cancer (PDAC) preclinical models different groups have shown the efficacy of both aspirin in combination with standard chemotherapy gemcitabine, and sulindac in prolonging survival in the Pdx1-Cre, LSL-$Kras^{G12D/+}$; LSL-$TrP53^{R172H/+}$ model of PDAC (Plassmeier et al. 2013; Li et al. 2013). Furthermore, an effect was observed on metastatic rate with sulindac, albeit in a small number of mice studied. Thus no longer are NSAIDs considered purely preventative, they may have therapeutic benefit particularly as an adjunct in combination with chemotherapy. Apricoxib, a novel COX2 inhibitor has recently been shown to enhance the efficacy of gemcitabine and erlotinib in orthotopic models of PDAC. Not only did addition of the NSAID enhance survival of mice but it also reduced metastases (Kirane et al. 2012). This effect seen on metastasis is similar to the reduction of metastatic disease observed in human studies (Rothwell et al. 2012b). Similarly when syngeneic orthotopic breast cancer cell lines were injected into the fat pads of adult mice, those treated with a COX2 inhibitor showed significantly fewer and smaller volume lung metastases, an action dependent on the M2 polarization of macrophages in the model (Retsky et al. 2012). Data obtained from patients treated with mastectomy for breast cancer has revealed that use of perioperative NSAIDs significantly improved disease free survival during the first 5 years. The use of NSAID ketorolac by inference appears to reduce the perioperative metastatic potential of the disease (Retsky et al. 2012).

Other agents readily available to practitioners today, including statins and bisphosphonates, exert COX independent anti-inflammatory actions and have been shown to be associated with reduced risk of cancer development in certain contexts (Park et al. 2014). Bisphosphonates for example, commonly used in both the treatment of osteoporosis and bone metastases, have recently been shown to reduce risk of colorectal cancer development in large series, interestingly the addition of statins did not further improve this relative risk improvement (Rennert et al. 2011). Statins may also have a role to play in downregulating inflammation in attempts to prevent cancer. Consistent downregulation of pro-inflammatory cytokines in cardiovascular trials has been demonstrated (Jain and Ridker 2005), this is supported by preclinical data that confirms inhibition of NF-κB signalling and pro-inflammatory cytokines in cancer models (Lee et al. 2007; Suh et al. 2011). The influence of statins on inflammation across all cancers is inconsistent, with one study showing no benefit in colorectal cancer development (Coogan et al. 2007).

17.3.1.2 Systemic Inflammation Confers a Poor Prognosis on Cancer Patients

The association between systemic inflammation, as denoted by C-reactive protein (CRP) levels, and outcome for patients following surgery has been studied in depth over the past decade across a range of tumour types (McMillan 2013). Our gauge for patient prognosis in cancer has always been pathological assessment of tumour stage. However, patient related factors, such as systemic inflammation and nutritional status are now recognised to influence outcome. Indeed, the correlation of rising CRP and falling albumin has been combined to provide a prognostic score in both operable and inoperable cancers independent of site (Forrest et al. 2003; McMillan 2013). Importantly, this work associates inflammation and cancer cachexia. Interestingly, very rarely is hypoalbuminaemia present in the absence of raised CRP suggesting the dependence of cancer cachexia on excess inflammation. Other authors have noted the impact systemic inflammation has on the response to therapy in cancer. By assessing blood neutrophil to lymphocyte ratios these authors were able to show that this measure of increased systemic inflammation impacted negatively on survival following chemotherapy in patients with malignant mesothelioma (Kao et al. 2010). Given the very clear association between inflammation and prognosis provided by these studies targeting inflammation as part of routine cancer therapy appears logical.

Of interest in this context is the FOCUS 4 trial in Colon cancer. Patients are molecularly subtyped following surgery and offered treatment based on the molecular subtype of their tumour. Patients may be excluded from therapy on the basis of a high platelet count, as a proxy for high levels of inflammation and poorer response to treatment (http://www.focus4trial.org/aboutfocus4/focus4summary).

17.3.2 Inflammatory Dysregulation as an Emerging Therapeutic Target

17.3.2.1 Failure of Tumour Immunosurveillance

In healthy individuals, the adaptive immune system is responsible for tumour immunosurveillance, through which the host can identify, mount a response to, and destroy tumour cells. Tumourigenesis represents a failure of adaptive immunity, since in order for malignant transformation to progress unchecked tumour cells must escape this surveillance. The immune response to cancer is a dynamic process and can also initiate pathways that are in fact pro-tumourigenic. Even then a fine balance exists, as tumour progression is complex, reliant on interactions between the tumour and its activated microenvironment (Baumgart et al. 2013). A number of immune cell types are involved in the development of the tumour microenvironment. Indeed many of these may be seen to exhibit dual roles. The immune cell infiltrate can be coordinated to eliminate tumour cells and play a role in adaptive immunity, while on the other side of the same coin can be co-opted by the tumour to permit tumour growth and progression. PDAC, as one of the most complex and stromally dense tumours, illustrates this balance particularly well (Fig. 17.1).

In early tumourigenesis, immune cells, including CD8+ T cells, detect danger signals from cancer cells and respond by eliminating these cells, leading to tumour immunogenicity. Dendritic cells are key regulators of tumour-specific immune responses in that they function as antigen presenting cells, activating CD8+ cytotoxic T lymphocyte (CTL) responses or stimulating CD4+ T cells through interaction with major histocompatibility complex (MHC) class II molecule-antigen complexes (Finn 2008). In pancreatic tumourigenesis, Hiraoka and colleagues reported that chemokine CXCL17 was responsible for infiltration of immature dendritic cells while ICAM-2 permitted killing of tumour cells by CD8+ cells. During evolution from precursor lesions to PDAC they found that the host immune response was tempered and tumours developed immune tolerance by downregulating CXCL17 and ICAM-2 (Hiraoka et al. 2011). Interestingly, Dillman and colleagues recently described the value of reintroducing dendritic cells loaded with antigen from autologous irradiated tumour cells in metastatic melanoma. Those patients who received this dendritic cell vaccine survived significantly longer than irradiated tumour cell vaccine alone (Dillman et al. 2011). Conversely, in PDAC, blockade of TLR4 signalling enhanced dendritic cell-mediated recruitment of T helper 2 (Th2) CD4+ cells, and this resulted in increased pancreatic inflammation and accelerated tumour progression (Ochi et al. 2012a). Thus depending on antigenic stimulus, dendritic cells can both promote and inhibit tumour progression. Promotion of antitumoural cell mediated responses, such as are generated through effective tumour immunosurveillance are being pursued in cancer. These strategies focus on engaging T cell responses in a field referred to as cancer immunology. The use of tumour antigen based vaccines and T cell adoptive transfer has provided some success in a small cohort of patients, however, the majority fail to respond clinically.

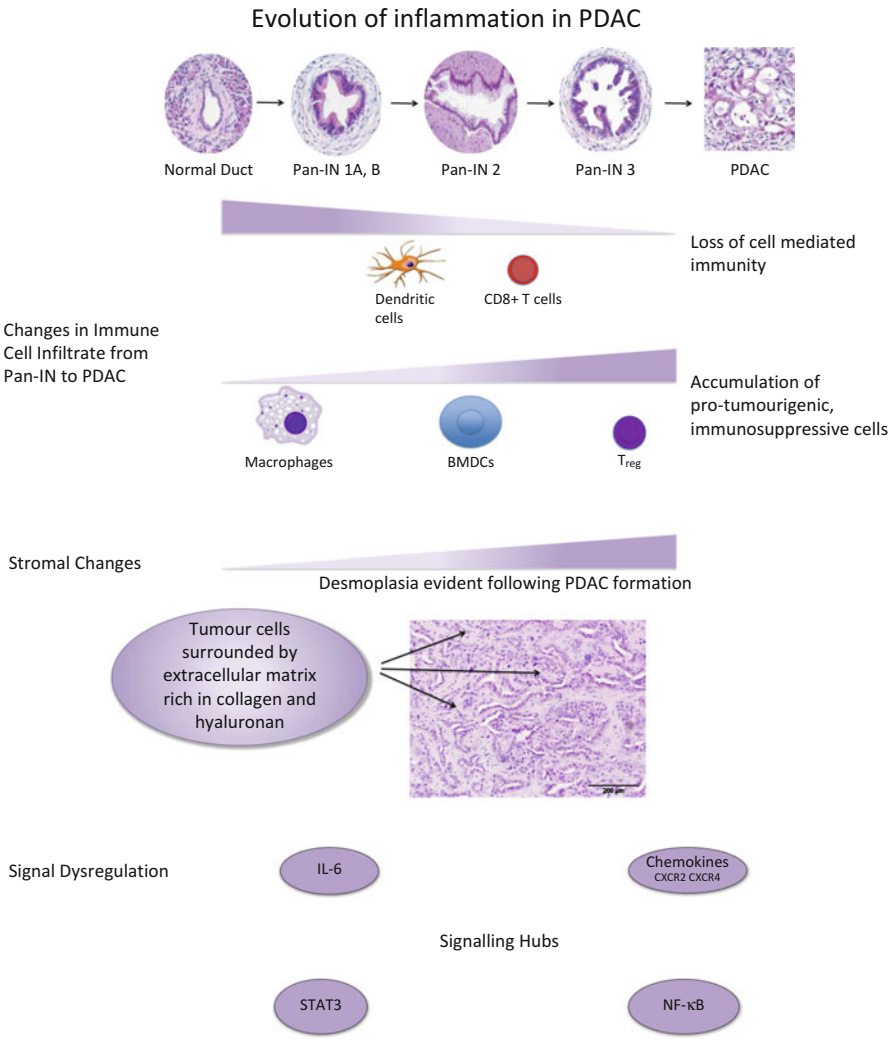

Fig. 17.1 Evolution of inflammation in pancreatic ductal adenocarcinoma (PDAC). Progression through pre-invasive pan-IN stages to PDAC is associated with loss of cell mediated immunity, decreased numbers of dendritic cells and CD8+ T cells and increased tumour associated macrophages and immunosuppressive bone marrow derived cells (BMDCs) and T regulatory cells (Treg). PDAC that forms is stromally dense, and thick with extracellular matrix proteins and up-regulated signalling pathways

More successful has been the use of T cell checkpoint inhibitors, to promote T cell activation. CTLA4 inhibitors such as ipilimumab and Programmed cell death 1 (PD1)/Programmed cell death ligand (PDL1) checkpoint inhibitors may be used to enhance CTL responses. Indeed the advances that these drugs have made in the field of metastatic melanoma have been striking. CTLA4 is a CTL surface protein and

functions as a negative regulator of T cell function. CTLA4 interacts with APC membrane bound co-stimulatory molecules and functionally competes with the T-cell co-stimulatory molecule CD28. Thus ipilimumab binds and inhibits CTLA4 releasing a break to T-cell co-stimulation. Ipilimumab was the first agent to prolong overall survival in metastatic melanoma. CTLA4 is not the only improvement in the understanding of checkpoint biology. PD1 is an inhibitory receptor which following chronic T cell activation is induced via ligands including PDL1. Production of PDL1 by tumour associated macrophages or tumour cells themselves permits immune evasion by this mechanism (Sullivan et al. 2013), thus making it an exciting target for therapy. Trials in melanoma thus far have shown reliable tumour responses in at least 50 % of cases.

Commonly difficulties with these types of therapies arise when there are insufficient T lymphocytes infiltrating tumours to activate. Indeed, certain authors have suggested that high T cell infiltrate denotes good prognosis in these patients, while low infiltrate suggests failure of this strategy (Gajewski and Schumacher 2013). Two major mechanisms of immune resistance appear to exist in this context: failure of T cell trafficking; and immune suppression within the microenvironment. It may be hypothesised that removal of immunosuppressive cells such as bone marrow derived cells (BMDCs) and macrophages from the tumour microenvironment may allow activation of a more sustained T cell response.

17.3.3 Inflammatory Tumour Microenvironment and Therapeutic Opportunities

17.3.3.1 Functions of the Cellular Component of Tumoural Inflammation, Using Pancreatic Cancer as an Example

Once tumour immune surveillance is overcome, the composition of the immune infiltrate changes and a pro-tumourigenic leukocyte profile emerges. It is not yet fully understood how these different pro and anti-tumoural components of the immune system are engaged. One important question without resolution at present is the degree to which the plasticity of certain immune cells can be manipulated for therapeutic gain, and whether these properties will hold across a number of different tumour types or remain tissue specific. Thus, the majority of the following data draws in the main from studies in PDAC, seemingly the most dense and active of all solid tumour microenvironments, and a tumour in which excellent murine models of disease have been established to permit its study.

Signals in the pancreatic tumour microenvironment, including IL-10, can prevent dendritic cell activation, thus dampening the adaptive immune response (Koido et al. 2010). T cells can be both tumour suppressing and promoting depending upon their downstream target cells. CD4+ cells, particularly T regulatory cells and Th2 cells increase in number over the course of pancreatic cancer progression, while CTL cells decrease (Clark et al. 2007). This dynamic circumstance leads to a

progressive accumulation of immunosuppressive cells that inhibit the anti-tumoural immune response. In tumours infiltrated by T regulatory cells both innate and adaptive immunity may well be suppressed, while BMDCs can also subdue anti-tumour activity (Gabrilovich and Nagaraj 2009).

In this milieu of tumour cell killing and escape, the immune system must clear damaged tissue. Neutrophils move into tissues under the control of chemokine signalling. Though short lived, neutrophils have the potential to induce significant tissue remodelling. Fridlender et al. demonstrated that following TGF-β blockade, neutrophils exhibited an anti-tumour phenotype termed N1 (Fridlender et al. 2009). Neutrophils are terminally differentiated cells, thus, they have often been overlooked with regards their role in cancer-related inflammation. However, neutrophils may also polarise to a pro-tumoural phenotype N2 in response to certain stimuli (Mantovani 2009), one of these stimuli is clearly TGF-β signalling. Chemokine receptor CXCR2 is expressed on neutrophils mediating chemotaxis of these cells to the tumour microenvironment (Jamieson et al. 2012).

Tissue macrophages evolve from blood monocytes, differentiating under the influence of signals received from the tissue microenvironment. Macrophages can be derived from existing populations initially established by the yolk sac during embryogenesis such as Kupffer cells in the liver. Macrophages following stimulation by bacterial lipopolysaccharides, and Th1 cytokines such as interferon gamma (IFNγ) adopt an M1 polarisation phenotype. M1 macrophages are broadly antimicrobial and anti-tumourigenic. Cytokine signals received from Th2 cells such as IL-13 and IL-4 drive an alternatively activated state, M2 macrophages, which largely carry out tissue remodelling purposes in health and following injury. These cells themselves express differing chemokine profiles. M1 macrophages produce pro-inflammatory chemokines including CXCL9 and CXCL10, in addition to IL-6 and TNFα among others following inflammatory stimuli, while M2 macrophages express non-inflammatory chemokines including CC chemokine ligand (CCL) 17, CCL18, CCL22, and CCL24 (Biswas et al. 2013). M2 macrophages fail to respond to inflammatory stimuli instead producing anti-inflammatory cytokines such as IL-10. It is possible for macrophages to exist on a spectrum between these two states, their functionality reliant on the pathophysiological stimulus.

Tumour-associated macrophages (TAMs) are present in early PDAC and persist throughout progression, representing an important component of the PDAC associated leukocyte infiltrate (Clark et al. 2007). Intriguingly, TAMs evolve to exhibit an M2 phenotype that is pro-tumourigenic in terms of promotion of growth and angiogenesis and suppression of adaptive immunity (Sica and Mantovani 2012). In cancer, this polarization of macrophages from a tumour-suppressive M1 phenotype may also be initiated by cytokine signals, including IL-10 and TGF-β, received from T regulatory and tumour cells (Sica and Mantovani 2012). This situation can be thought of as self-reinforcing, since production of pro-angiogenic molecules, including vascular endothelial growth factor (VEGF), attracts yet more macrophages to the tumour microenvironment, further enhancing angiogenesis.

Colony stimulating factor (CSF) 1 and its receptor CSF1R act as master regulators of macrophage differentiation from myeloid precursors. Via production of CSF1 and

other chemokines including CCL2, tumours are able to chemo-attract macrophages to the tumour microenvironment (Hamilton and Achuthan 2013). CCL2 overexpression has been associated with progressive macrophage accumulation and progression of disease for some time now (Lin et al. 2001). CCL2 has also been identified as important in the recruitment of Ly6C+ macrophages from blood to sites of metastasis (Qian et al. 2011). The origin of TAMs is a matter of some debate, with the potential of both recruitment from blood and in situ proliferation possible. Indeed, much of the literature recently has pointed towards infiltration of BMDCs of a Ly6C+ subtype from the spleen to the tumour microenvironment resulting in macrophage differentiation in situ (Movahedi et al. 2010). The plasticity of TAMs makes them an ideal therapeutic target for manipulation in order to generate an anti-tumour response, as potentially does neutrophil directed therapy as described above.

There are examples where TAMs have been seen to elicit cancer destructive properties. Hagemann and colleagues have suggested that macrophages may be re-educated towards an anti-tumour function through control of NF-κB activity (Hagemann et al. 2008). Trabectedin is a recently approved chemotherapeutic that binds DNA and in so doing blocks the cell cycle affecting gene transcription and DNA repair. Importantly selected monocyte toxicity and inhibition of both CCL2 and IL-6 in vitro suggest a potential role in targeting macrophages in tumours (Germano et al. 2013). Three models of transplantable tumours, fibrosarcoma, lewis lung cancer, and ovarian carcinoma were assessed in vivo. There was a significant inhibition in each of these models of blood and splenic monocytes following trabectedin treatment. Furthermore, trabectedin treatment decreased tumour-associated macrophage numbers, a process dependent on caspase 8 activation. These findings highlight the potential for ablation of certain immunosuppressive cell types and the potential advantage of combinatorial approaches, for example anti-VEGF inhibitors whose efficacy is negated by TAMs. Measures to harness the immune system to eradicate tumour cells are being considered. An excellent example was recently provided in a cohort of metastatic PDAC patients who received CD40 targeting monoclonal antibodies in addition to gemcitabine (Beatty et al. 2011). CD40 activation, somewhat unexpectedly led to macrophage-dependent tumour regression. Promotion of properties of the adaptive immune system that protect us from malignancy in health holds promise for future trials.

Given recent evidence that immature myeloid cells referred to as BMDCs, classified as Gr1+, Cd11b+, Ly6C/Ly6G+ cells, have a profound effect on tumour immunosurveillance, and appear largely responsible for differentiation into both neutrophils (Ly6G+) and macrophages (Ly6C+), perhaps myeloid precursors themselves are the ideal targets for therapy in cancer. The progressive accumulation of BMDCs in PDAC is widely recognized, while their immunosuppressive effect is clear. They are known to have the capacity to differentiate into both neutrophils and macrophages (Kusmartsev et al. 2005), and it has been suggested that protumourigenic N2 neutrophils (TANs) may differentiate from BMDCs of splenic origin. Indeed two groups have demonstrated that in response to oncogenic Kras dependent granulocyte, macrophage colony stimulating factor (GM-CSF) secretion, BMDCs are recruited to the tumour microenvironment where they play a role in

suppressing CTL cell action and thus permitting tumourigenesis (Pylayeva-Gupta et al. 2012; Bayne et al. 2012). When GM-CSF was suppressed, BMDCs failed to infiltrate the tumour microenvironment and tumour growth was limited by infiltrating CTL cells. Furthermore, this effect was fully rescued by CTL depletion. Activated neutrophils also have the capacity to cause tissue damage via release of MMPs, while production of reactive oxygen species may promote mutagenesis. Reactive oxygen species have been implicated in the activation of NF-κB, which has been intimately associated with the inflammatory response and PDAC progression (DeNicola et al. 2011). Indeed the apparent importance of BMDCs in the mediation of immunosuppression and avoidance of surveillance raises their potential manipulation in concert with drugs that enhance T cell effectiveness permitting a CTL response. Regulation of T cell responses is mediated by both Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and PD1 checkpoints. Expression of ligands to these receptors on the surface of both myeloid cells and tumour cells can provide an off signal to the receptors and therefore failure to mount T cell response to cancer. Thus therapies that inhibit myeloid cell differentiation and chemotaxis to the tumour microenvironment are being combined with T cell activation strategies to attempt to overcome these complexities of the tumour microenvironment.

17.3.3.2 Stromal Signalling Pathways

In addition to immune cells other stromal elements play key roles in PDAC pathogenesis. Paracrine signalling through molecules including TGF-β, VEGF, hepatocyte growth factor, sonic hedgehog (Shh), epidermal growth factor, fibroblast growth factors and insulin-like growth factors all signal to the adjacent microenvironment, and in particular PSCs. This interaction activates and, is primarily responsible for fibrosis in PDAC (Neesse et al. 2011). They also implicated in local tumour growth in addition to their ability to travel to distant sites and support metastatic formation (Neesse et al. 2011). Elevated levels of factors secreted into the microenvironment including SPARC, a protein associated with cell migration and wound healing, have been associated with poor outcome in PDAC (Infante et al. 2007). Interestingly albumin-bound Paclitaxel (nab-paclitaxel) has been observed to bind SPARC-expressing fibroblasts, possibly providing a mechanism for targeting this specific cell type. Clinical trial of nab-paclitaxel in PDAC has shown some promise in combination with gemcitabine (Von Hoff et al. 2011), however the full role of SPARC in modulating the tumour microenvironment is unclear, with few changes seen in tumour stroma when preclinical PDAC models were treated (Frese et al. 2012).

The dense, avascular, collagenous extracellular matrix that constitutes the majority of the tumour bulk in human PDAC has been shown to mechanically block the vascular delivery of chemotherapeutic agents to tumours in preclinical models (Neesse et al. 2011). It is anticipated that drugs that attack stromal elements responsible for tumour maintenance will be of clinical benefit. Thus far, clinical trials assessing anti-stromal therapies including anti-MMP and VEGF inhibitors

such as Bevacizumab have proven disappointing (Neesse et al. 2011). Among others, Shh signalling through Smoothened (Smo) has been implicated in the coordination of tumour-stromal crosstalk in PDAC. The clinical trial based on the work of Olive and colleagues (2009), who showed modest prolongation of survival of mice with PDAC following Smo inhibition in combination with gemcitabine, has been stopped due to better survival in the control arm (clinicaltrials.gov). However, there remains a belief that combating collagenous elements of PDAC stroma and promoting intratumoural vascularity will help delivery of standard chemotherapeutics to tumour cells. Tumours are dependent on both the protective effect of the stroma and interactions with stromal cells for progression. Hyaluronan has been identified as a key determinant of the stromal matrix with hyaluronidase treatment causing profound effects on tumour stroma in murine models of PDAC (Jacobetz et al. 2013; Provenzano et al. 2012). These drugs have also been shown to expand tumour vasculature and permit access of chemotherapeutics to the pancreas providing promise for ongoing clinical trials.

17.3.4 Emerging Importance of Inflammation in Metastasis

Metastasis, a hallmark of cancer, can be conceptualised as a multi-stage process where neoplastic cells spread from the tumour of origin and colonise distant sites. Local invasion, intravasation, survival in the circulation, extravasation and colonisation of the metastatic site are all required for development of metastases (Hanahan and Weinberg 2000). Therefore, the process of metastasis requires the coordination of a number of different biological processes. Pancreatic cancer sufferers die primarily as a result of tumour burden and commonly with metastatic disease despite surgical resection and adjuvant therapy. Indeed metastasis is the source of approximately 90 % of cancer deaths (Hanahan and Weinberg 2000) and is the major source of death in PDAC (seercancergov/statfacts/html/pancreas). In these patients, tumour cells either disseminate early in the course of PDAC, and colonisation takes longer to establish than primary tumours meaning these lesions are undetectable by preoperative imaging, or, tumours disseminate late near to the point at which diagnosis is made and therefore colonisation goes undetected with current imaging modalities. There may be a period of colonization therefore, that allows a window of therapeutic opportunity following early diagnosis of the primary tumour.

The process of metastasis can be considered in the context of the ‘seed and soil’ hypothesis first proposed by Paget in 1889. This hypothesis suggests that microenvironmental elements are required by tumour cells (the seed) at distant sites (the soil) to permit establishment of metastatic disease.

Recent studies have shown metastases may occur in PDAC even before a primary tumour has formed confirming early dissemination, a behaviour associated with early epidermal mesenchymal transformation (Rhim et al. 2012). This process was accelerated in the presence of pancreatic inflammation, while the most invasive areas of tumour were seen at foci of inflammation. This phenotype was suppressed

by dexamethasone, highlighting the integral role played by tumoural inflammation. NF-κB has been implicated in the process linking inflammation and cancer progression (Ling et al. 2012), however, it is likely that multiple different inflammatory pathways are implicated in this process including STAT-3 (Lesina et al. 2011), TLR4 signalling (Ochi et al. 2012b) and CXCR2 signalling (Ijichi et al. 2011).

'Preconditioning' of metastatic sites by non-cancerous immune cells including macrophages and BMDCs has been described (Barcellos-Hoff et al. 2013) in order to create a receptive soil for metastasis. It is thought that this pre-metastatic niche is established by such primary tumour components even prior to the presence of tumour cells at metastatic sites (Wels et al. 2008). BMDCs may cooperate with other molecules such as fibronectin, tenascin and MMP9 to develop a receptive environment for tumour establishment. It is believed that the production of chemokines and growth factors by primary tumour cells stimulate production of this environment. It has been suggested that CXCL1 production by breast cancer cells is important in metastasis formation in experimental models, implicating CXCR2 signalling in the establishment of the metastatic niche. However, full explanation of how CXCR2 signalling is implicated in the metastatic process in other cancers remains in its infancy. It is appreciated that primary lung cancer cells may produce VEGF-A, TGF-β, and TNFα resulting in upregulation of S100A8/9 in the premetastatic lung (Acharyya et al. 2012). S100A8/9 can be expressed by both neutrophils and macrophages (Passey et al. 1999). Furthermore it has recently been shown that progenitor BMDCs, particularly of the myeloid lineage, are responsible for upregulation of these molecules (Sade-Feldman et al. 2013). TLR4 signalling may also be important in the crosstalk between BMDCs and tumour cells during the process of metastatic niche formation (Barcellos-Hoff et al. 2013).

Macrophages have been described as 'obligate partners for metastasis' (Condeelis and Pollard 2006). Indeed genetic knockout of CSF1 in a mouse model of breast cancer almost completely abrogated metastases by interfering with macrophage homing (Lin et al. 2001). It has been shown that histidine-rich glycoprotein is important in suppressing the M2 protumourigenic phenotype of macrophages to a tumour inhibiting M1 phenotype. Blockade of placental growth factor was important for this phenotype. These effects suppress pro-angiogenic effects of macrophages and result in tumour vessel normalisation (Rolny et al. 2011). In addition vascular cell adhesion molecule (VCAM)-1 can tether breast cancer cells to tumour-associated macrophages during the development of lung metastases protecting the cells from immune cell destruction (Chen et al. 2011). These data provide an insight into the potential of tumour associated immune cells to influence cancer cell chemotaxis, invasion, and protection from immune destruction however, substantial investigation is required to clarify the underlying molecular mechanisms of these processes.

Extracellular components have been implicated in the process of metastasis. Lysyl oxidase (LOX) is a copper-dependent amine oxidase required for the covalent crosslinking of collagen. Significant associations between LOX and tumour progression have been observed, with high expression in breast, colorectal and head and neck cancers among others. In particular, LOX expression has been associated

with hypoxia in tumors. Indeed in areas of hypoxia induction of LOX by hypoxia inducible factor has been observed to mediate metastases in vivo (Erler et al. 2006). Importantly LOX has been seen to play a critical role in pre-metastatic niche formation. LOX secretion by metastatic breast cancer cells cross-linked collagen IV to which BMDCs adhered releasing MMP2 to digest the matrix and allow influx of metastasising tumour cells (Erler et al. 2009).

PSCs are normally resident in the pancreas, and are responsible for the stromal reaction of PDAC. When quiescent they are characterised by vitamin A containing lipid droplets in their cytoplasm. These cells when activated lose this characteristic and become myofibroblast-like in morphology and function. They secrete an extracellular matrix component that forms fibrous tissue (Apte et al. 2004). There is crosstalk between PDAC cells and PSCs as has been demonstrated by the potential of PDAC cells to stimulate proliferation, migration and activity of PSCs in vitro (Bachem et al. 2005). Meanwhile PSCs are able to promote the survival of cancer cells through the inhibition of apoptosis (Vonlaufen et al. 2008). This highlights another way in which PDAC harnesses stromal cells to facilitate progression. PSCs are important in the development of the metastatic niche in PDAC. Using an orthotopic model of PDAC using human cancer cells mixed with human PSCs Xu et al. demonstrated that primary tumours were significantly larger and more stromal dense in the presence of PSCs (Xu et al. 2010). The metastatic rate was significantly higher in mice where PSCs were present within the orthotopic pancreatic injection group, and as PSCs were cultured from male patients and injected into female mice, fluorescence in situ hybridisation for the Y chromosome was able to demonstrate that PSCs from the primary site had indeed infiltrated secondary sites with the tumour cells. As yet few therapies have sought to target the signalling axis between PSCs and tumour cells.

17.4 Conclusions

In conclusion the myriad potentially activated inflammatory pathways across different cancers suggest that targeting of one pathway will seldom be sufficient to improve outcome, particularly in cancers such as PDAC. However, combination of a number of strategies, with chemotherapy may both improve chemotherapy efficacy and interfere with tumour-stromal crosstalk that mediates disease progression (Fig. 17.2). There are a number of well-characterised pathways through which the majority of tumoural inflammation is mediated. Many of these pathways have been shown to be important in tumour progression. Attractive targets include: general anti-inflammatories which target COX and other important inflammatory mediators; enhancement of cell mediated anti-tumoural immune response via reprogramming antigen presenting cells such as dendritic cells; activation of T cell mediated immune responses; removal of immunosuppressive BMDCs and macrophages from the tumour microenvironment; and interfering with commonly activated chemokines and transcription factors in pro-tumoural inflammation such as IL-6, TNFα,

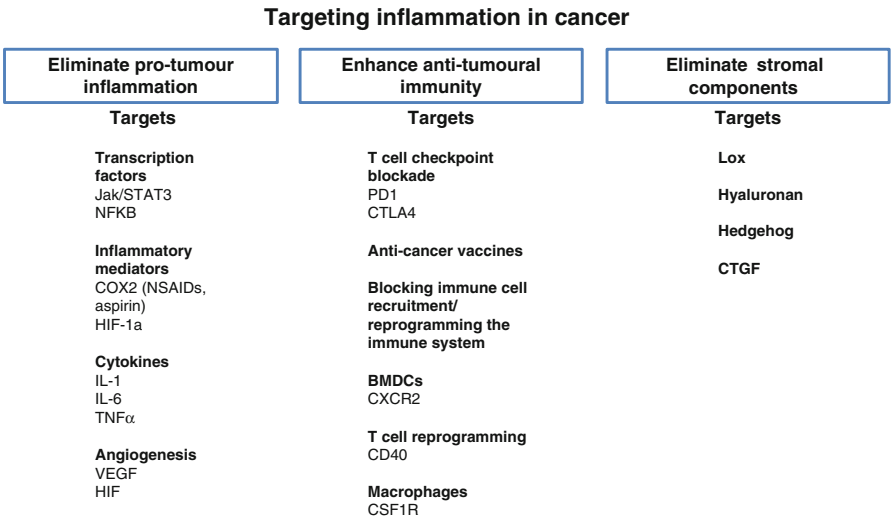

Fig. 17.2 Targeting inflammation in cancer. Strategies to eliminate cancer via inflammatory modulation: Eliminate pro-tumoural inflammation; enhance anti-tumoural immunity; eliminate stromal components

STAT3, and NF-κB. There is evidence from preclinical work that in combination with conventional chemotherapies these anti-inflammatory therapeutic approaches may activate scavenging effector systems as are seen in remodelling of wounds during healing (Coussens et al. 2013). At present there exists promising preclinical data regarding use of anti-inflammatory therapies in cancer. There are a few clear success stories such as the use of T cell checkpoint inhibitors in metastatic melanoma that provide hope for a large number of cancer patients in the future. However, only in the coming decade will we be able to judge the success of translation of these therapies from bench to bedside across different cancers. With such promising pre-clinical data, the time has now come for the translation to patient therapy with the aim of revolutionising cancer care and survival.

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Chapter 18
Cell-Nonautonomous ER Stress-Mediated Dysregulation of Immunity by Cancer Cells

Jeffrey J. Rodvold, Navin R. Mahadevan, and Maurizio Zanetti

Abstract The immune surveillance hypothesis posits that neoantigens presented by tumor cells are detected by the immune system and eliminated, keeping tumor formation and growth at bay. Operationally this requires that tumor cells be taken up by local sentinels of the immune system, myeloid antigen presenting cells, which then proceed to present tumor associated antigens to T cells, resulting in specific rejection of tumor cells. Yet, one of the central unsolved paradoxes of tumor immunology is how the tumor escapes immune control which is reflected in the lack of effective autochthonous or vaccine-induced anti-tumor T cell responses.

In this chapter we discuss the emerging new idea that the endoplasmic reticulum (ER) stress response/unfolded protein response (UPR) activated in response to tumor microenvironmental *noxae*, acts not only as a key cell-intrinsic regulator of tumor growth and survival, but also as a central cell-extrinsic modulator of myeloid cell and T cell function. We will review the cellular and molecular basis of the anti-tumor immune response and the polarization of myeloid cells and T cells and place these into a UPR-centered perspective. We will also present the UPR as a cell-extrinsic regulator of anti-tumor immunity, effected by the newly-discovered "transmissible" ER stress.

Keywords ATF6: Activating transcription factor 6 • CHOP: CCAAT/-enhancer binding protein homologous protein • DC: Dendritic cell • ECM: Extracellular matrix • eIF2α: Eukaryotic translation initiation factor 2 alpha • EMT: Epithelial to mesenchymal transition • ER: Endoplasmic reticulum • FOXP3: Forkhead box 3 • GRP78: Glucose regulated protein 78 • HSR: Heat shock response • IDO: Indoleamine 2,3-dioxygenase • IL-: Interleukin • iNOS: Inducible nitric oxide synthase • IRE1α: Inositol requiring enzyme 1 alpha • LAG3: Lymphocyte activation gene 3 • MDSC: Myeloid derived suppressor cell • MEF: Mouse embryonic fibroblast • MHC: Major histocompatibility complex • NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells • OVA: Ovalbumin • PD-L1: Programmed cell death ligand 1 • PERK: Protein kinase-like endoplasmic reticulum kinase • PGE2: Prostaglandin E2 • TAM: Tumor associated macrophage • TCR: T-cell receptor • TERS: Transmissible endoplasmic

J.J. Rodvold • N.R. Mahadevan • M. Zanetti (✉)
Department of Medicine and Moores Cancer Center, The Laboratory of Immunology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0815, USA
e-mail: mzanetti@ucsd.edu

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reticulum stress • TGFβ: Transforming growth factor beta • TIDC: Tumor infiltrating dendritic cell • TLR: Toll like receptor • UPR: Unfolded protein response

18.1 Introduction

Modern tumor immunology takes its roots in Burnet's immune surveillance hypothesis, which posits that the immune system is able to recognize tumor-associated antigens and act as a cell-extrinsic regulator of tumor growth (Burnet 1970). In humans, the immune surveillance hypothesis is supported by the detection of naturally-occurring T cell responses against self tumor antigens (Yotnda et al. 1998a, b; Molldrem et al. 2000; Nagorsen et al. 2003; Filaci et al. 2006), suggesting that central tolerance does not completely delete precursor T cells specific for a variety of self tumor antigens from the available repertoire. Studies in mice on sporadic cancer initiated through the rare spontaneous activation of a dormant oncogene showed that these tumors are in fact immunogenic and do not escape recognition by T cells but rather induce tolerance associated with the expansion of non-functional T cells (Willimsky and Blankenstein 2005). This is consistent with the observation that CD8 T cells generated by vaccination in melanoma patients are functionally heterogeneous and have a predominantly quiescent phenotype (Monsurro et al. 2002, 2004), reflecting perhaps a defective activation during priming. Thus, the complex landscape of anti-tumor T cell response depends on a delicate balance between activation of the residual T cell repertoire specific for self tumor antigens and mechanisms controlling the state of activation and function of T cells against these antigens.

Recently, emphasis has been placed on loss of immune surveillance subsequent to the disruption of the equilibrium at the tumor/immune interface mediated by tumor infiltrating myeloid cells (Balkwill and Mantovani 2001; Serafini et al. 2006). Virtually all solid tumors (carcinomas most notably) contain infiltrates of diverse leukocyte subsets including both myeloid- and lymphoid-lineage cells (Tlsty and Coussens 2006). Tumor-infiltrating leukocytes are rich in CD11b+ myeloid cells (Serafini et al. 2006), subsets of which produce factors that promote tumorigenesis by acting on tumor cells and immune cells. These secreted molecules include inflammatory cytokines that promote tumor cell growth and survival (IL-6, IL-23, and TNF-α, (Langowski et al. 2006; Kim et al. 2009) and for review see (Grivennikov et al. 2010; Mumm and Oft 2008), but also suppressive factors that inhibit T cell responses (e.g., IL-10, TGFβ, arginase – (*Arg1*), and indoleamine 2–3 dioxygenase (IDO)) (for review see (Gabrilovich et al. 2012)). Thus, tumor cells and the immune cells within the tumor microenvironment utilize "pro-inflammation" and "suppression" to exact a toll on adaptive T cell responses and facilitate tumor escape and growth (Van Ginderachter et al. 2006). However, the exact link between microenvironmental pro-inflammation/suppression and impairment of T cell function is not clearly understood.

Recent data from this laboratory provide a unifying view for this complex interplay, linking tumor cells, their microenvironment, leukocyte infiltration,

inflammation, and immune suppression. Our interpretative framework is based on the observation that the endoplasmic reticulum (ER) stress response in cancer cells causes the release of a factor(s) that itself recapitulates both ER stress in myeloid cells, macrophages and dendritic cells (DC), polarizing them to a pro-inflammatory/suppressive phenotype, ultimately impairing fundamental processes of the adaptive T cell response (Mahadevan et al. 2011a, 2012).

The endoplasmic reticulum (ER) is the initial checkpoint for the folding and modification of proteins that reside within the secretory pathway. The ER stress response, or unfolded protein response (UPR), is mediated by three initiator/sensor transmembrane molecules, inositol-requiring enzyme 1α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which, in the unstressed state, are maintained in an inactive state through association with 78 kDa glucose-regulated protein (GRP78) (Walter and Ron 2011). Upon ER stress induction, GRP78 disassociates from the three UPR sensors, de-repressing them and allowing downstream signaling. Upon activation, PERK signals downstream effectors such as the growth arrest and DNA damage gene (GADD34) and the C/EBP homologous protein (CHOP), a regulator of inflammatory gene transcription and apoptotic cell machinery (Walter and Ron 2011). IRE1α is an endoribonuclease that, upon activation, initiates the unconventional splicing of the mRNA encoding X-box-binding protein 1 (XBP-1). Spliced XBP-1 is a potent transcriptional activator that increases expression of a subset of the UPR-related genes involved in efficient protein folding, maturation, and degradation in the ER (Lee et al. 2003b).). In addition, under ER stress or forced autophosphorylation, IRE1α's RNase can cause endonucleolytic decay of many ER-localized mRNAs through a phenomenon termed regulated IRE1-dependent decay (RIDD) (Walter and Ron 2011). The transcription factor ATF6 activates UPR target genes that ameliorate the protein folding capacity of the ER (Walter and Ron 2011).

Cancer cells are consistently exposed to ER stress-inducing *noxae* within the tumor microenvironment. These include nutrient deprivation due in part to chaotic vasculature and highly active nutrient (i.e. glucose) consumption (aerobic glycolysis) (Warburg 1956), an imbalance between demand and supply of oxygen (hypoxia), an imbalance between the production of reactive oxygen and the cell's ability to readily detoxify the reactive intermediates (oxidative stress), and aberrant glycosylation (Heazlewood et al. 2008). Chronic viral infections (He 2006) which account for 18 % of cases of cancer world-wide (WHO International Agency for Cancer Research), als o cause ER stress.

It is the objective of this chapter to draw attention to the emerging paradigm that the cell-extrinsic consequences of tumor-borne UPR influence the dynamic equilibrium that exists at the tumor / immune interface. Specifically, we will discuss the anti-tumor immune response; its subversion via the co-existence of inflammation and suppression in the tumor microenvironment; the cell-intrinsic role of the UPR tumor adaptation and survival; cell-extrinsic mechanisms of tumor immune evasion; the UPR-mediated cell-extrinsic dysregulation of myeloid cells with impairment of antigen presentation and CD8 T cell activation; and therapeutic approaches targeting the tumor UPR.

18.2 The Anti-tumor Immune Response and Subversion

That immunity plays a protective role against spontaneous tumors dates back to 1912 and the pioneering work of G. Schone (cited in (Silverstein 1989)). In modern times this idea formed the basis of Burnet's immune surveillance hypothesis (Burnet 1970, 1971). New experimental data have provided for a revision of the original immune surveillance hypothesis (Schreiber et al. 2011) suggesting that tumor cell variants may not be completely eliminated by the immune system but instead enter into an equilibrium phase during which T cells constrain clinically undetectable occult tumor cells and edit tumor cell antigenicity and immunogenicity (Koebel et al. 2007). The resulting edited tumor cells possess reduced immunogenicity and begin to grow in an immunologically unrestrained manner.

18.2.1 The Anti-tumor Adaptive Immune Response

Adaptive anti-tumor T cell responses are based on the recognition of antigens expressed on the surface of tumor cells in association with molecules of the major histocompatibility complex (MHC). However, self tumor antigens use a variety of strategies to evade immune surveillance: tolerance/anergy, ignorance and active immunosuppression through soluble mediators. In addition, escape also occurs through immune suppression mediated by CD4 and CD8 regulatory T cells (Tregs) (Sakaguchi 2003; Sakaguchi et al. 2008), a class of cells increased in patients with malignancies and in tumor tissues (Zou 2006; Mougiakakos et al. 2010; Jacobs et al. 2012; Whiteside 2012). Studies in mice show that antigen specific tumor-infiltrating CD8 T lymphocytes display an activated phenotype but little cytotoxicity when transferred into tumor-bearing mice (Savage et al. 2008). Sporadic tumors in mice are immunogenic but induce tolerance associated with the expansion of non-functional T cells (Willimsky and Blankenstein 2005). T cells tolerant to self antigen return to a tolerant phenotype even after having resumed proliferation and function (Schietinger et al. 2012). This shows that tumor-initiated *active* regulation of the adaptive T cell response plays an important role in the lack of effectiveness of anti-tumor immunity.

18.2.2 Tumorigenic Cytokines in the Tumor Microenvironment

Inflammatory cytokines, often under the control of NF-κB, promote tumor cell survival, proliferation, and immune subversion. While oncogene activation in tumor cells can lead to cytokine production and secretion, the predominant source of tumorigenic inflammatory mediators are tumor-infiltrating myeloid cells (Grivennikov et al. 2010). For example, inhibition of NF–κB by ablation of IKKβ

in liver macrophages results in loss of TNF-α and IL-6 production, which in turn, impairs tumor growth (Pikarsky et al. 2004). Macrophage-specific deletion of IKKβ leads to decreased production of PGE_2 and IL-6, resulting in reduced incidence of colitis-associated colorectal tumors (Greten et al. 2004). Myeloid cells (macrophages and dendritic cells) of the lamina propria were found to be a key source of IL-6, which plays a crucial role in driving tumorigenesis in a mouse model of colitis-associated cancer (Grivennikov et al. 2009). In a model of lung cancer, IL-6 and TNF-α produced by myeloid cells in response to tumor-derived versican (Kim et al. 2009) drive tumor growth and progression in a TLR2-dependent manner. IL-6 production by hepatocellular carcinoma (HCC) progenitor cells and Kupffer cells in early dysplastic lesions in a model of carcinogen-driven liver carcinogenesis promotes progression to HCC (Naugler and Karin 2008; Akers et al. 2013).

IL-23 produced by tumor associated macrophages (TAMs) blocks CD8 T cell infiltration into skin tumors (Langowski et al. 2006) and upregulates T regulatory cell differentiation in the melanoma microenvironment (Kortylewski et al. 2009). In a mouse model of spontaneous colon cancer, bacterial TLR ligands penetrate the colonic mucosal barrier and promote IL-23 production by adenoma-infiltrating myeloid cells, ultimately leading to tumor outgrowth likely via induction of downstream tumor-promoting cytokines, including IL-17 and IL-6 (Grivennikov et al. 2010). In addition, tumor-associated myeloid dendritic cells are a key source of IL-23 in a mouse model of lung cancer, and a neutralizing IL-23 antibody combined with agonistic CD40 antibodies reduces primary fibrosarcoma and metastatic melanoma tumor burden (von Scheidt et al. 2014).

The TGFβ family of cytokines, has different roles at different stages of tumorigenesis within in the tumor microenvironment. The source of TGFβ can be tumor cells themselves, especially early in tumor growth; however infiltrating myeloid cells are a major TGFβ source later during tumor progression (reviewed in (Massague 2008)). Early during tumor growth, TGFβ restrains tumorigenesis via cell-intrinsic and cell-extrinsic mechanisms: (1) repression of the cell cycle and induction of cell cycle inhibitors, (2) promotion of cellular differentiation and senescence, (3) activation apoptotic machinery, (4) suppression of autocrine and paracrine mitogenic signaling in neighboring stromal fibroblasts, and (5) inhibition of innate and adaptive immune cell function and tumorigenic cytokine production (reviewed in (Massague 2008; Pickup et al. 2013)).

During tumor progression, however, malignant cells inactivate TGFβ signaling and can co-opt other tumorigenic functions of TGFβ signaling, including extracellular matrix (ECM) degradation via matrix metalloproteinase production, epithelial-to-mesenchymal transition (EMT) (Chaffer and Weinberg 2011), and stimulation of angiogenesis. In this context, TGFβ can promote tumorigenic inflammatory and immunosuppressive effects in invading immune cells. For instance, TGFβ and IL-6 drive CD8 and CD4 T cell differentiation to the Tc17 and Th17 phenotypes, which promote tumor growth in the correct context via promotion of angiogenesis and tumor cell proliferation (reviewed in (Pickup et al. 2013). Inversely, TGFβ signaling directs polarization of tumor-associated myeloid cells to a suppressive phenotype, which inhibits T cell function *in vitro* and perhaps *in vivo* (reviewed in (Pickup et al.

2013; Mao et al. 2014)). In addition, TGFβ signaling in CD8+ T cells represses expression of the Natural killer group 2, member D (NKG2D) receptor and inhibits cytolytic activity (Friese et al. 2004; Thomas and Massague 2005).

It should be noted that, while the tumorigenic role of various inflammatory mediators, including NF-kB, IL-6, IL-23, and TGFβ, have been well illustrated, the tumor-mediated mechanism of their production in the tumor microenvironment, notably, by tumor-associated myeloid cells, remains less clear.

18.2.3 Tumor-Associated Myeloid Cells

Virtually all solid tumors (carcinomas most notably) contain infiltrates of diverse leukocyte subsets, mainly myeloid cells (Tlsty and Coussens 2006), which express the CD11b+surface marker (Serafini et al. 2006; Ruffell et al. 2012) and have been stratified into tumor-associated macrophages (TAM) ($F4/80^{+}/Gr1^{+}$), myeloid-derived suppressor cells (MDSC) ($Gr\text{-}1^{+}$) and tumor infiltrating myeloid dendritic cells ($CD11c^{+}$). As a whole, myeloid cells that infiltrate solid tumors are key players in the cell-extrinsic regulation of tumor growth, often producing a variety of pro-tumorigenic factors that effectively modify the tumor/immune cell landscape. Because of their ability to inhibit T cell responses *in vitro* and *in vivo* (Kusmartsev et al. 2004; Huang et al. 2006), and the initial characterization of their phenotype as $IL\text{-}10^{+}/IL\text{-}12^{-}$ coupled with low levels of costimulatory molecules and antigen presentation machinery, it was proposed that tumor-associated CD11b+/Gr1+ myeloid cells possessed an anti-inflammatory and suppressive (M2) phenotype (Mantovani et al. 2002). Tumor infiltrating dendritic cells (TIDC) were first characterized as having an immature phenotype characterized by low levels of MHC Class I and II, and co-stimulatory molecule (CD86/CD80) expression, which was assumed to be responsible for the dysfunctional T cell priming and induction of anergy observed by immature DC in non-tumor systems or in the peripheral blood of cancer patients (Chaux et al. 1997; Gabrilovich et al. 1997; Probst et al. 2003; Friese et al. 2004).

More recently, however, evidence has accumulated that suggests that the tumorigenic phenotype of myeloid cells is concomitantly pro-inflammatory and actively suppressive (for a extensive review on the topic, see (Ostrand-Rosenberg and Sinha 2009)). For instance, in tumor-associated myeloid cells, generation of reactive oxygen species crucial for the inhibition of T cell responses can occur via arginase, a classical M2 marker, but also via iNOS, an inflammatory (M1) marker (Otsuji et al. 1996; Kusmartsev et al. 2004). Furthermore, tumor-derived myeloid cells produce inflammatory cytokines that play key roles in tumor growth and in regulating anti-tumor immunity (Mumm and Oft 2008; Grivennikov et al. 2010). More recently, it has been found that TIDC in melanoma, lung carcinoma, ovarian cancer, and breast cancer express high levels of MHC Class I/II, CD80, and CD86, yet they still inhibit anti-tumor CD8 T cell responses *in vitro* and *in vivo* due to a combination of inadequate antigen presentation, arginase production, or PD-L1 expression (Stoitzner et al. 2008; Liu et al. 2009; Norian et al. 2009; Engelhardt

et al. 2012; Scarlett et al. 2012). For example, in a murine model of ovarian carcinoma, as well as in human ovarian tumor samples, TIDC with a "regulatory" phenotype hallmarked by expression of MHC II, CD86, and DEC205, promote tumor outgrowth by suppressing T cell function within the tumor via IL-6 activity, PD-L1, Arginase I, respectively (Scarlett et al. 2012; Tesone et al. 2013).

Large cohort studies in breast cancer patients have shown that the presence of macrophages expressing CD68 correlates with poor prognostic features (Denardo et al. 2011), increased angiogenesis (Cavanagh et al. 2005) and decreased disease-free survival (Cairns et al. 2011). In addition, presence of increased numbers of CD68+ macrophages in tumor stroma in patients with non-small-cell lung carcinoma (NSCLC) correlated with poorer overall survival (Welsh et al. 2005; Kawai et al. 2008; Dai et al. 2010).

18.3 Co-existence of Inflammation and Suppression in the Tumor Microenvironment and the Cell-Intrinsic Contribution of the UPR to Tumor Progression

There is increasing evidence that the tumor/immune interplay is important in tumor growth and invasiveness (Hanahan and Weinberg 2011), and that local inflammation (Balkwill and Mantovani 2001; Balkwill et al. 2005; Grivennikov et al. 2010; Cou ssens et al. 2013) plays a key role. The vast majority (95 %) of cancers display, and likely result from, somatic, as opposed to germline mutations (Vogelstein et al. 2013). Since inflammation has been linked to genomic instability (Tili et al. 2011), inflammation-promoting conditions (obesity, diet, stress, viruses) could serve as precondition to cancer growth and progression. In addition, infiltrating myeloid cells and T cells have the capacity to produce a variety of pro-tumorigenic factors that effectively modify the tumor/immune cell landscape. For example, tumorigenic effects have been associated with pro-inflammatory cytokines (IL-6, IL-23, TNF-α and MIF), but, also with anti-inflammatory cytokines (IL-10, TGFβ) and molecules with immune suppressive function (arginase, peroxynitrite and indoleamine 2–3 dioxygenase) (for review see (Gabrilovich et al. 2012)). Furthermore, through the secretion of a variety of cytokines, tumor-infiltrating myeloid cells also contribute to tumor angiogenesis (Shojaei et al. 2007; Kujawski et al. 2008; Chen and Bonaldo 2013).

How myeloid cells become causative for tumor growth and progression and what tumor-derived cues determine their polarization is still poorly understood. Even more perplexing is the apparent paradox that the tumor microenvironment is at once pro-inflammatory and anti-inflammatory, suggesting the possibility that myeloid cells may, at a certain point, possess a "mixed" pro-inflammatory/suppressive phenotype (Van Ginderachter et al. 2006). Hereunder we will present evidence for a unifying mechanistic interpretation of this paradox.

18.3.1 Cell-Intrinsic Role of UPR in Tumor Adaptation and Progression

The starting point of our new hypothesis is evidence implicating ER stress and the UPR in tumorigenesis, cancer growth, and progression. Primary human tumor cells of several origins, including breast (Fernandez et al. 2000), lung (Uramoto et al. 2005), liver (Shuda et al. 2003), colon (Xing et al. 2006), prostate (Daneshmand et al. 2007), and brain (Pyrko et al. 2007) have been shown to upregulate UPR pathways, whereas peritumoral areas do not. Additionally, in primary human melanoma, liver, and breast cancer specimens, the level of GRP78 positively correlates with tumor progression (Fernandez et al. 2000; Shuda et al. 2003; Zhuang et al. 2009). GRP78 has also been detected on the surface of tumor cells of diverse histological origin (Arap et al. 2004; Davidson et al. 2005; Misra et al. 2006).

The conditional homozygous knockout of *Grp78* in the prostate of mice with *Pten* inactivation protects against cancer growth (Fu et al. 2008) and inactivation of a *Grp78* allele in the *MMTV-PyT* murine model of breast cancer yields significantly decreased breast tumor proliferation, survival, and angiogenesis compared to *Grp78*+/+, *PyT* mice (Dong et al. 2008). Lastly, proliferating and dormant cancer cells in which *Grp78* is upregulated are resistant to chemotherapy (Reddy et al. 2003; Ranganathan et al. 2006; Chang et al. 2007; Fu et al. 2007; Pyrko et al. 2007).

Transformed mouse fibroblasts deficient in *Xbp1* are more sensitive to hypoxic stress *in vitro* than wild type cells, and do not grow as tumors when injected into SCID mice. Consistent with these findings, mouse embryonic fibroblasts (MEFs) expressing a siRNA against *Xbp-1* lead to tumors that are smaller and exhibit decreased angiogenesis as compared to tumors generated by control cells when injected into mice (Romero-Ramirez et al. 2004, 2009). Similarly, siRNA inhibition of *Xbp-1* in human fibrosarcoma cells inhibits their growth and angiogenesis in a xenograft model, and overexpression of XBP-1s in human fibrosarcoma cells expressing a dominant-negative IRE1α mutant rescues xenograft angiogenesis (Romero-Ramirez et al. 2004, 2009). Additionally, human glioma cells expressing a dominant-negative IRE1α mutant display a decreased growth rate and impaired angiogenesis when orthotopically transplanted into immunodeficient mice (Drogat et al. 2007).

The inactivation of PERK or a dominant-negative PERK in tumor cells, results in tumors that are smaller and less aggressive than their normal counterparts when implanted into mice (Bi et al. 2005). And finally, tumor cells cultured under hypoxic/anoxic conditions and transformed cells in hypoxic areas of tumors activate ER stress. Inactivation of PERK results in impaired tumor cell survival under hypoxic conditions *in vitro*, and decreased tumor growth and angiogenesis *in vivo* (Bi et al. 2005; Blais et al. 2006). Taken together, these results underscore the key contribution of UPR in the adaptation and progression of solid tumors of diverse origins.

18.3.2 UPR Involvement in Regulation of Inflammatory Mediators

Besides promoting cellular adaptation to increased un/misfolded protein burden, the UPR activates a pro-inflammatory cascade with tumor-promoting and cell-survival effects. One of the key inflammatory regulators inducible by the UPR is the transcription factor NF-κB (Bonizzi and Karin 2004). Each of the three UPR signaling pathways activates NF-κB translocation to the nucleus via distinct mechanisms. PERK-mediated translational inhibition reduces the ratio of the IκB to NF-κB thus permitting the nuclear migration of NF-κB and transcription of downstream inflammatory genes (Jiang et al. 2003; Deng et al. 2004). Upon auto-phosphorylation, IRE1α forms a complex with tumor-necrosis factor-α (TNF-α)-receptor-associated factor 2 (TRAF2) at its cytosolic domain, and the IRE1α-TRAF2 complex mediates direct IκB phosphorylation via IκB kinase (IKK), which leads to NF-κB activation (Hu et al. 2006). Lastly, ATF6 was shown to participate in NF-κB activation in an AKT-dependent manner (Yamazaki et al. 2009).

The UPR is linked to the production of several inflammatory, tumorigenic cytokines: IL-6, IL-23, and TNF-α. A microarray analysis of mouse lymphoma cells under *in vitro* pharmacological ER stress reveals transcriptional upregulation of multiple inflammatory genes, including *Il-6*, *Il-23p19*, *Tnf-α*, *Tlr2*, and *Cebpb* (Wheeler et al. 2008). Furthermore the levels of *in vivo* ER stress, as measured by *Grp78* expression, correlate with *Il-6*, *Il-23p19*, and *Tnf-α* transcription in murine prostate cancer cells growing in a heterotopic transplantation model (Mahadevan et al. 2010).

CHOP is necessary for IL-23 production by dendritic cells (Goodall et al. 2010), and IL-6 and TNF-α by macrophages (Chen et al. 2009). Redundant roles for IRE1α and PERK signaling in IL-6 and TNF-α production in macrophages have been reported (Chen et al. 2009; Martinon et al. 2010). ChIP analysis also reveals that XBP-1s binds to the promoters of the *Il-6* and *Tnf-α*; congruently, Ire1α- or *Xbp1-deficient* macrophages display impaired IL-6 and TNF-α production in response to pharmacological ER stress and infectious TLR agonism (Martinon et al. 2010). The UPR also synergizes with TLR4 agonism to result in robust IL-23 secretion by macrophages (DeLay et al. 2009). Interestingly, murine tumor-associated macrophages deficient in the UPR effector chaperone Grp94, have reduced inflammatory cytokine (IL-1β, IL-6, IL-17A, IL-17F, IL-18, IL-22, IL-23, IFN-γ, and TNF-α) production, which correlates with decreased colitis-associated tumor burden (Morales et al. 2014). Thus, the UPR is a key regulator of the production of inflammatory mediators.

18.3.3 UPR-Mediated Dysregulation of Antigen Presentation

In addition, evidence suggests that UPR signaling in tumor cells and in antigen-presenting cells can impinge upon antigen presentation. While B cells mount a UPR following accumulation of a KDEL-retained protein in the ER upregulate

MHC Class II and costimulatory molecules, they present decreased levels of high affinity peptide complexed to MHC Class II (Wheeler et al. 2008). 293T cells overexpressing an ER stress-inducing misfolded protein or constitutively active ATF6 or XBP-1s display decreased levels of MHC Class I (de Almeida et al. 2007). Similarly, mouse thymoma cells that undergo ER stress through palmitate treatment or glucose deprivation decrease antigen presentation on MHC Class I (Granados et al. 2009). Induction of UPR genes in lymphoma cells with thapsigargin or the histone deacetylase inhibitor, trichostatin A, is associated with the transcriptional downregulation of tapasin (Pellicciotta et al. 2008; Wheeler et al. 2008), a chaperone molecule involved in quality control of MHC I/peptide complexes in the ER (Howarth et al. 2004). Moreover, IRE1α-mediated signaling upregulates the expression of miR346, which in turn downregulates the protein transporter associated with antigen processing 1 (TAP1), ostensibly decreasing MHC Class I-associated antigen presentation (Bartoszewski et al. 2011). Most recently, it was shown that in CD8α$^+$ dendritic cells, IRE1α regulated dependent decay (RIDD) of mRNAs coding for components of the MHC Class I presentation pathway, including tapasin, leads to dysfunctional cross presentation and cross-priming of antigen-specific CD8+ T cells (Osorio et al. 2014). These findings suggest that cancer cells and antigen presenting cells mounting a UPR undergo remodeling of the processing machinery yielding decreased presentation of high affinity immunodominant peptides.

18.4 Cell-Extrinsic Polarization of Myeloid Cells via the Tumor UPR

Recent reports have begun suggest that the UPR can regulate anti-tumor immunity via modulation of myeloid cell function. For instance, hyperploid cancer cells translocate the ER chaperone, calreticulin, to the cell membrane in a UPR-dependent manner, promoting tumor cell phagocytosis by myeloid antigen presenting cells, ultimately possibly initiating a cellular immune response against hyperploid cancer cells (Senovilla et al. 2012).

On the other hand, we uncovered a previously unappreciated cell-extrinsic effect of the tumor UPR: its transmission to myeloid cells, i.e., macrophages and DC (Mahadevan et al. 2011b, 2012), which culminates in subversion of anti-tumor immunity. This new phenomenon, "transmissible" ER stress (TERS), was discovered while investigating the effects of conditioned medium from ER stressed murine tumor cells (e.g., prostate, melanoma, and lung carcinoma) on bone marrow-derived macrophages and dendritic cells (DC). In these experiments, cancer cells were stressed using thapsigargin, a sesquiterpene lactone canonical ER stress inducer that inhibits the sarco/endoplasmic reticulum Ca^{2+} ATPase, or glucose starvation. We found that bone marrow-derived macrophages and DC both function as receivers of TERS. The changes imparted on receiver cells include (i) the induction of a global ER stress response (e.g., the transcriptional upregulation of *Grp78*, *Xbp-1s*, and

Chop); (ii) the upregulation of pro-inflammatory/pro-tumorigenic cytokines (i.e., *Il-6*, *Il-23p19*, and *Tnf-α*); (iii) the increased secretion of other pro-tumorigenic cytokines/chemokines (TGFβ, MIP-1α, MIP-1β); and (iv) the upregulation of Arginase 1 (Mahadevan et al. 2011b, 2012), an enzyme that suppresses T cell function (Bronte and Zanovello 2005). TERS had no effect on IL-10 in myeloid cells. In addition, TERS-imprinted myeloid cells do not upregulate GR-1, distinguishing their phenotype from that of classical MDSC (Gabrilovich et al. 2012). We found that PD-L1, the ligand for the T cell immune-inhibitory PD-1 receptor (Freeman et al. 2000), is somewhat increased by TERS in myeloid DC (Mahadevan et al. 2012). TERS-imprinted myeloid DC rapidly change morphology, acquiring morphological characteristics of activated DC, including increased size and elongated dendrites. They also upregulate expression of MHC Class I and Class II, and the costimulatory molecules CD86, CD80 (classical markers of immune activation), and, to a lesser extent, CD40 (Mahadevan et al. 2012). (Cullen et al. 2013) confirmed that TERS from breast cancer cells can remodel macrophage phenotype similar to the pro-inflammatory/suppressive one described above, and, in addition, demonstrated that TERS-imprinted macrophages secrete VEGF. The general phenotypic features of $CD11b^{+}$ cells, macrophages and DC, upon TERS imprinting are summarized in Fig. 18.1. In addition, transmission of ER stress was reduced in TLR4KO macrophage receiver cells, suggesting that TLR4 senses, at least in part, the transmission of tumor borne stress.

Taking into account the current evidence, the cell-extrinsic effects of the tumor UPR appear to be contrasting: on the one hand promoting immune surveillance of

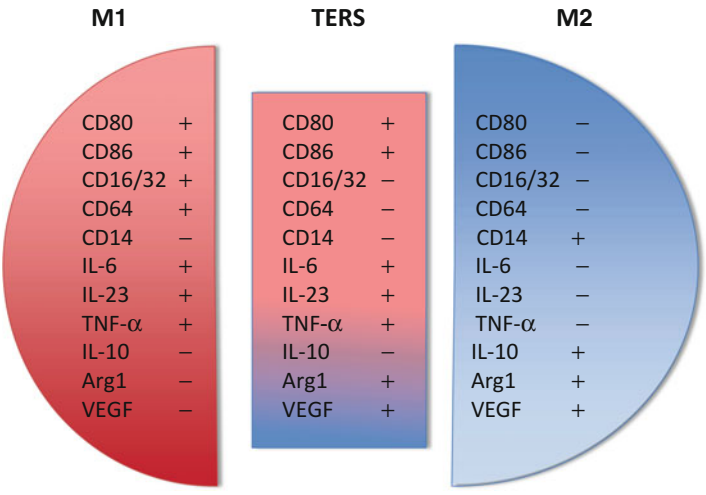

Fig. 18.1 The pro-inflammatory/suppressive phenotype of TERS-imprinted myeloid cells. The characteristics of TERS-imprinted bone marrow-derived macrophages (*middle*) are compared with those of canonical characteristics of M1 (*left*) and M2 (*right*) macrophages (Adapted from Zanetti (2013))

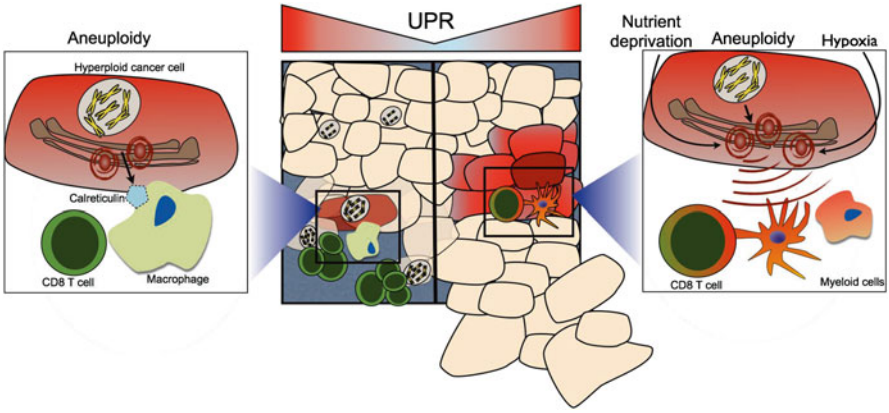

Fig. 18.2 Janus-faced effects of the tumor UPR on anti-tumor T cell immunity. (*Left and left inset*) Hyperploid cancer cells induce a UPR-dependent translocation of calreticulin to the cell surface where it serves as a "eat-me" signal for phagocytic cells (macrophages and dendritic cells). These take up tumor cell debris and apoptotic bodies, and present tumor antigens to T cells, inducing an anti-tumor immune response, which leads to the selective elimination of hyperploid cancer cells (Senovilla et al. 2012). (*Right panel and right inset*) The tumor UPR polarizes infiltrating myeloid cells to a pro-inflammatory/suppressive phenotype characterized by inefficient antigen presentation and CD8 T cell cross-priming, ultimately derailing anti-tumor T cell immunity, leading to tumor growth facilitation (Mahadevan et al. 2012) (From Mahadevan et al. (2013) with permission)

hyperploid tumor cells via ER stress-enforced calreticulin expression (Senovilla et al. 2012), while, on the other, imprinting macrophages and dendritic cells with a pro-inflammatory/suppressive, mature phenotype with functional abnormalities with respect to antigen processing and presentation to T cells, as will be discussed below. To reconcile these seemingly contrasting effects, we suggested (Mahadevan et al. 2013) that the tumor UPR response may fulfill both functions, perhaps promoting cellular immunity against hyperploid cells on the one hand early during tumorigenesis, while ultimately undermining the immune response against cancer cells. That clinical tumors samples exhibit heterogeneous ploidy (Ohyama et al. 1990), and tetraploidy is key event in the progression of diverse histological subtypes (Davoli and de Lange 2011), suggest that this might indeed be the case. The seemingly Janus-faced cell-extrinsic role of the tumor UPR is shown in Fig. 18.2 (adapted from (Mahadevan et al. 2013)).

18.5 TERS Inhibits Antigen Presentation and CD8 T Cell Priming by Bone Marrow-Derived DC

In a series of experiments, we demonstrated that TERS impacts adversely upon myeloid DC cross-presentation and cross-priming (Mahadevan et al. 2012), two events associated with the induction of CD8 T cell-mediated immunity.

18.5.1 Effects on Cross-Presentation

To study cross-presentation we used a system in which the ovalbumin (OVA) SIINFEKL peptide bound to the H2-K^b molecule can be detected by flow cytometry using a monoclonal antibody. Reproducibly, OVA-fed, TERS-imprinted DC have reduced display of the SIINFEKL/H2-K^b complex at the cell surface, while the expression of MHC Class I molecules remains constant or even increased over that of OVA-fed control DC. Thus, TERS down-regulates cross-presentation.

18.5.2 Effects on Cross-Priming/T Cell Activation

To study cross-priming we used CD8 T cells from OT-I mice whose T cell receptor (TCR) is specific for the SIINFEKL/H2-K^b complex. In this model, OVA-fed bone marrow-derived DC efficiently induce both the activation and proliferation of OT-I CD8 T cells. When OT-I CD8 T cells are co-cultured with OVA-fed TERS-imprinted bone marrow-derived DC, however, while being activated, they proliferate poorly. On average, the majority (>70 %) of CD8 T cells are activated/non-dividing T cells. PD-1, a marker of "exhausted" T cells, is not upregulated. Importantly, antigen-specific CD8 T cells activated by TERS-imprinted myeloid DC show decreased ability to kill target cells [our unpublished data].

Importantly, we found that TERS-imprinted bone marrow-derived DC could exert dominant suppression over cross-priming by normal bystander antigen presenting cells. When TERS-imprinted bone marrow-derived DC, with or without antigen, are added to co-cultures naïve CD8 T cells and antigen-fed control bone marrow-derived DC, the proliferation of CD8 T cells is suppressed.

18.5.3 Mechanisms of Cross-Priming Defect

Efforts to restore the proliferative defect showed the following. (i) The addition of excess SIINFEKL antigen (1 μg/mL), rescued proliferation in OT-I T cells cross-primed by TERS-imprinted bone marrow-derived DC. (ii) The addition of exogenous IL-2 during cross-priming failed to rescue OT-I T cell proliferation, ruling out the possibility of classical anergy (Beverly et al. 1992). (iii) Removal from the co-culture containing TERS-imprinted bone marrow-derived DC partially restored T cell proliferation, although with fewer cell divisions, suggesting the importance of cell-cell contact. (iv) Whereas the addition of exogenous L-arginine to the co-culture did not improve T cell proliferation, the addition of L-norvaline, a competitive inhibitor of arginase, rescued it in great part (80 %). Taken together, these results suggest that tumor UPR-mediated myeloid cell-derived arginase activity and impaired cross-presentation together contribute to the T cell proliferative defect

observed. Interestingly, however, addition of L-norvaline did not rescue T cell proliferation caused by dominant suppression.

18.5.4 TERS-Imprinted Myeloid DC in Context

Modeling the cell-extrinsic influence of the tumor UPR showed that TERS-imprinted BMDC are phenotypically mature, upregulate costimulatory molecules, have diminished cross-presentation capacity, and exert suppressive activity over CD8 T cells and bystander DC. Tolerogenic DC have been described in various systems (Steinman et al. 2003), and were initially defined in the periphery as steady-state, immature cells able to present antigen that suppress T cell activity because of inadequate co-stimulatory capacity (Gabrilovich et al. 1997; Steinman and Nussenzweig 2002). In the microenvironment of solid tumors of several histological types, infiltrating dendritic cells can be identified that display an immature phenotype with decreased MHC Class II, CD80, CD86, and CD83 expression, with presumed passive T cell inhibitory activity (Chaux et al. 1997; Bell et al. 1999; Pinzon-Charry et al. 2005; Tesone et al. 2013).

However, evidence has begun to accumulate ascribing active immunosuppressive activity via several mechanisms (e.g. arginase, IDO, and PD-L1 activity; for review, see (Tesone et al. 2013)) to phenotypically mature, so-called "regulatory" dendritic cells (Tesone et al. 2013). TERS-imprinted myeloid DC recapitulate *ab initio* several characteristics of these cells, including increased CD80, CD86, PD-L1, MHC Class II, and arginase activity with decreased antigen presentation capacity (Stoitzner et al. 2008; Liu et al. 2009; Norian et al. 2009; Scarlett et al. 2012; Engelhardt et al. 2012; Tesone et al. 2013). Given that regulatory dendritic cells have been isolated from epithelial cancers prone to a microenvironmental UPR, it is possible that the tumor UPR is a key modulator of myeloid antigen presenting cell, and ultimately, T cell function. A comparison of TERS-imprinted myeloid-derived dendritic cells with TIDC in different murine experimental systems and human patients is presented in Table 18.1.

18.6 Implications of TERS-Directed Cross-Priming on Fate Determination of CD8 T Cells

Initial lineage analysis of $CD8^+$ T cells cross-primed by TERS-imprinted bone marrow derived DC showed transcriptional upregulation of the cytokines *Il-10* and *Tnf-* α but not *Il-17*, upregulation of *Foxp3*, and downregulation of the costimulatory molecule CD28. LAG3, a negative costimulatory molecule (Huard et al. 1994) found on tumor-infiltrating T cells (Grosso et al. 2007), was slightly up-regulated. When we analyzed the 96-h TERS-imprinted myeloid DC:T cell co-culture supernatant, we observed increased secretion of IL-2 but no elevation of IL-10,

Table 18.1 Comparison of different types of tumor associated myeloid cells and their effects on T cells

Type of cells	Mechanism of origin	Effect on CD8 T Cells	Mechanism of action	CTL Function	Ref.
MDSC (Gr1+)	Unknown	Inhibit naïve cell expansion	Immature DC	ND	(Kusmartsev et al. 2004)
			TCR nitration		
TuDC (CD11c+)	Unknown	Inhibit naïve cell expansion	Arginase and rescued by norvaline	ND	(Norian et al. 2009)
		Dominant suppression			
TuDC (CD11c+)	Unknown	Inhibit naïve cell expansion and Ag experienced cell re-stimulation	Ag experienced CD8 T cells rescued by TLR7/TLR9 ligands	Decreased	(Engelhardt et al. 2012)
Reg DC (MHC II/ CD11c+/CD86+)	Tumor-derived PGE2, TGF-β1	Inhibit tumor Ag experienced T cell re-stimulation	PD-L1, arginase?	ND	(Scarlett et al. 2012)
		Dominant suppression			
BMDC* (CD11c+)	Transmissible UPR	Inhibit naïve cell expansion	Reduced Ag presentation	Decreased	(Mahadevan et al. 2012)
		Dominant suppression	Arginase and rescued by norvaline		

**BMDC* bone marrow-derived dendritic cells

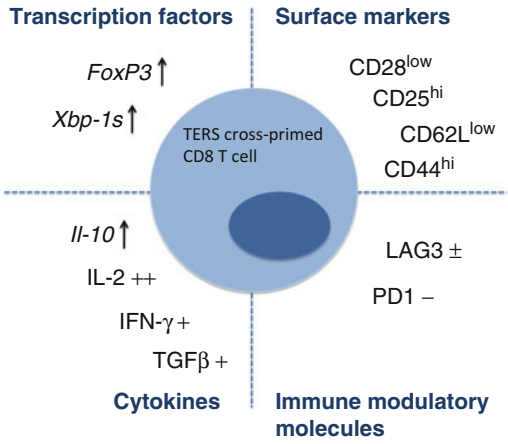

Fig. 18.3 Synopsis of phenotypic characteristics of murine CD8 T cells cross primed by TERS-imprinted myeloid DC

IL-17, IFN-γ or TNF-α compared to control (Fig. 18.3). A provisional conclusion is that CD8 T cells cross-primed by TERS-imprinted bone marrow-derived DC display an uncommitted phenotype with potential suppressive characteristics (CD28 downregulation and *Il-10* upregulation) (Filaci et al. 2007). Surprisingly, $CD8^+$ T cells cross-primed by TERS-imprinted BMDC also demonstrated disproportionately high splicing of *Xbp-1* compared to only modest upregulation of other UPR elements, the significance of which remains unknown.

In sum, the phenotype of CD8+ T cells cross-primed by TERS-imprinted myeloid DC appears similar to that of $CD8^+/CD28^-$ regulatory T cells secreting IL-10 and TNF-α, and expressing FOXP3, which have been found to infiltrate a variety of human tumors (Becker et al. 2000; Kruger et al. 2001; Filaci et al. 2007; Mahic et al. 2008). It still remains to be seen whether, like human CD8 suppressor T cells, TERS-directed CD8 T cells have suppressor functions effected by surface ecto-ATPases (e.g. CD39) and/or soluble mediators (e.g. IL-10). A comparison of the CD8+ T cell phenotype derived from TERS-imprinted APC with CD8+T cells infiltrating human tumors is presented in Table 18.2.

18.7 The Effect of TERS *In Vivo* and Mechanism of Generation

Several lines of evidence suggest that TERS is operational *in vivo*. First C57BL/6 mice injected intra-peritoneally with TERS develop an ER stress response in liver cells characterized by the up-regulation of *Grp78*, *Chop* and spliced *Xbp-1*. This suggests that a tissue that is sensitive to ER stress induction, the liver, readily becomes a target of TERS administered systemically.

Table 18.2 Comparison of tumor-associated CD8 regulatory T cells

Immune phenotype	Species	Mechanism of Origin	Inhibitory Effects	Mechanism of Action	Ref.
CD8+/ CD28-/ CD45RA+/ CCR7-/ FOXP3-/CD56-	Human	Soluble factors (IL-2, IL-10, TGF-b, GM-CSF)	Suppression of allogeneic CD8 and CD4 T cell expansion	CD39	(Fenoglio et al. 2008; Filaci et al. 2007; Parodi et al. 2013)
		Agnon-specific *in vitro*		IL-10	
CD8+/ CD45RO+/ CCR7+/ IL-10+	Human	Tumor-associated plasmacytoid DC	Suppression of Ag-specific and allogeneic T cell activation	IL-10?	(Wei et al. 2005)
CD8+/ CD28-/ FOXP3+	Mouse	TERS-imprinted myeloid DC (arginase-dependent)	Impaired CTL [a]function	ND[b]	(Mahadevan et al. 2012)

[a]*CTL* cytotoxic T lymphocytes
[b]*ND* not determined

18.7.1 TERS-Imprinted Myeloid Cells Promote Tumor Progression In Vivo

The effect of TERS on tumor growth was examined in C57BL/6 mice inoculated subcutaneously with B16.F10 tumor cells admixed with TERS-imprinted bone marrow-derived DC according to Prehn (1972). Under these conditions, we noted an earlier tumor initiation, accelerated tumor growth, and decreased survival when compared to mice receiving B16.F10 tumor cells admixed with control bone marrow-derived DC, or tumor cells alone (Mahadevan et al. 2012). Thus, bone marrow-derived DC polarized by ER-stressed tumor cells facilitate tumor growth *in vivo*. B16.F10 tumors seeded with TERS-imprinted bone marrow-derived DC contained about half the percentage of tumor infiltrating $CD8^+$ T cells as compared with control B16.F10 tumors. Interestingly, while we found a decreased number of CD8 T cells in tumors, we found no difference in the draining lymph nodes, implying the local nature of this phenomenon.

TERS-imprinted bone marrow-derived DC also function to dysregulate anti-tumor T cell immunity, allowing immune escape. For instance, TC1.OVA prostate cancer cells that constitutively express OVA, which functions as a tumor rejection antigen (Redmond et al. 2007), do not form tumors in mice reflecting their immunogenic status. However, when inoculated admixed with TERS-imprinted bone marrow-derived DC, they form transient tumors 6–10 days post-injection (Mahadevan et al. 2012).

18.7.2 *TERS Is Produced* In Vivo *During Tumor Formation*

New evidence shows that tumor-infiltrating myeloid cells *in vivo* display TERS characteristics. CD11b^{+} myeloid cells isolated from B16.F10 tumors implanted in C57BL/6 mice, or from spontaneous intestinal adenomas in *adenomatous polyposis coli* (*APC*) mice, display both an upregulation of the UPR and the mixed pro-inflammatory/suppressive phenotype typical of the TERS signature compared with bone marrow- or spleen-derived myeloid cells from tumor-bearing mice (Rodvold et al. 2014b).

18.7.3 *TERS Requires Ire1α Signaling in Transmitter Cells*

Because the ER stress response is under the control of three main sensors (IRE1α, PERK, ATF6), we began to deconvolute their precise role in the generation of TERS. Using MEFs each deficient in a single arm of the UPR, we found that compared with wild type MEFs the production of TERS was greatly diminished in IRE1α KO MEFs but not in PERK KO or ATF6 KO MEFs, providing the first indication that TERS generation may be mainly due to IRE1α signaling (Rodvold et al. 2014b). A hypothetical model of the signaling events involved in the generation of TERS is illustrated in Fig. 18.4.

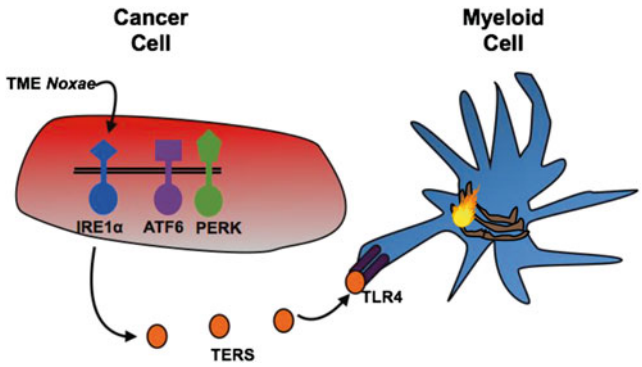

Fig. 18.4 Hypothetical model for the generation of TERS in transmitter cells. The model predicts that of the three main UPR sensors (PERK, ATF6 and IRE1α), Ire1α is responsible for the generation/release of the TERS factor(s) from stressed transmitter cancer cells. Myeloid cells (macrophages and dendritic cells) receive TERS factor(s), which is sensed in part by TLR4, and are polarized to a phenotype characterized by activation of the UPR (flame) and a pro-inflammatory/suppressive phenotype that facilitates tumor growth (see text for details)

18.8 Therapeutic Approaches Targeting the Tumor UPR

In the previous sections we discussed the cell-intrinsic role of the UPR in tumor adaptation and survival, as well as its putative cell-extrinsic role in polarizing myeloid antigen presenting cells to a phenotype that facilitates tumor outgrowth via T cell-dependent and independent mechanisms. Considering this dual role, targeting the UPR in the tumor microenvironment will likely have a dual benefit: impairing tumor cell microenvironmental adaptation and survival, and disabling a mechanism of host immune subversion. Based on our current understanding, the cellular targets, of any such intervention would be the tumor cell, myeloid antigen presenting cells, and CD8+ T cells (Fig. 18.5a). It remains to be seen whether CD4 T cell immunity is also adversely affected by the cell-extrinsic effects of the UPR.

The UPR is tumor microenvironment-specific as demonstrated by studies showing that peritumoral areas do not express UPR genes and that a constitutive UPR takes place within spontaneously growing tumors, though heterogeneously within a tumor mass (Spiotto et al. 2010). In addition, several lines of evidence indicate that UPR inhibitors selectively target tumor cells, as discussed below.

As the UPR represents an adaptive mechanism to cope with ER stress, targeting the UPR will likely take the following forms: (1) inhibition of the UPR in tumor cells with high levels of basal ER stress (eg. microenvironment-driven: hypoxia, glucose deprivation; tumor-intrinsic: secretory tumors, like myeloma), or (2) exacerbation of ER stress and consequent induction of cytotoxic/apoptotic effects. While each of these approaches will individually exploit tumor microenvironmental ER stress, either by its induction *or* by targeting its adaptive response (the UPR), they alone may not be sufficient to control the UPR within the complex and heterogeneous tumor microenvironment. For instance, exacerbating ER stress alone may exhibit cytotoxicity, especially in hypoxic/nutrient deprived areas; however, tumor cells mounting a UPR that leads to survival will have a UPR-mediated adaptive advantage, including resistance to chemotherapy (Pyrko et al. 2007) and host immunity. On the other hand, only inhibiting the UPR will target tumor cells with increased basal ER stress due to heterogenous microenvironmental *noxae*, sparing cells in more vascularized areas. We propose that optimal targeting of the UPR should take the form of inducing ER stress in tumor cells (fueling the fire) while concomitantly inhibiting the UPR (locking up the extinguisher) (Fig. 18.5b). In sum, this combinatorial mechanism will simultaneously take advantage of the cytotoxic potential of ER stress while inhibiting the response mechanism needed to adapt.

These strategies have already gained some experimental support. Bortezomib, a proteasome inhibitor that induces accumulation of proteins thus exacerbating ER stress, causes significantly higher cytotoxicity in hypoxic HeLa and human colorectal cancer cells than in normoxic cells, an effect dependent on ER protein load and consequent ER stress (Fels et al. 2008). Similarly, the induction of ER stress with a

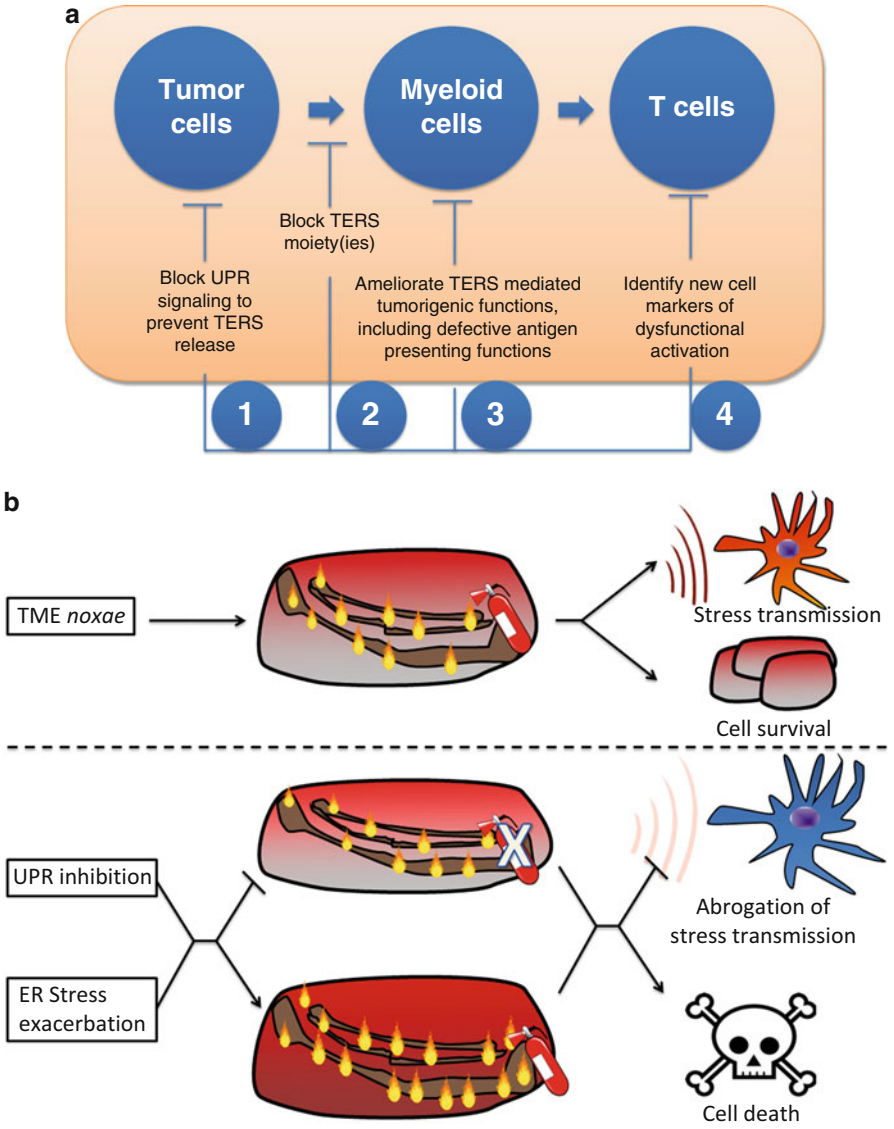

Fig. 18.5 Targeting the cell-extrinsic effects of the UPR in the tumor microenvironment. **(a)** Points of therapeutic intervention against the tumor-promoting effects of TERS. **(b)** Strategies for pharmacological control of the UPR in the context of cancer. (*upper panel*) Before any intervention, cancer cells exhibit a basal level of unfolded proteins (flames) compensated by the UPR (fire extinguisher). A combinatorial approach should involve tuning down the UPR (*middle panel*) with simultaneous exacerbation of ER stress (*lower panel*), hence inducing cell death in cells unable to cope with ER stress, as well as inhibiting the negative cell-extrinsic effects of the tumor UPR

targeted thapsigargin pro-drug, celecoxib, or bortezomib, induces glioblastoma cell death, especially in hypoxic areas of the tumor (Johnson et al. 2002; Denmeade et al. 2012; Schonthal 2013). Combination of the ER stress inducers bortezomib and celecoxib, or its non-coxib analogue, 2,5-dimethyl-celecoxib (DMC), causes severe ER stress and apoptosis in murine glioblastoma cells *in vitro* and *in vivo* (Kardosh et al. 2008).

Inhibition of *Xbp-1* splicing in multiple myeloma with the IRE1α endoribonuclease small molecule inhibitor, STF-083010, results in tumor cell-specific death *in vitro* and *in vivo* (Papandreou et al. 2011). Similarly, irestatin, a small molecule inhibitor of IRE1α endoribonuclease activity, inhibits hypoxic human myeloma and colon cancer cell survival and colony formation *in vitro*, as well as *in vivo* tumorigenesis in a heterotopic xenograft model (Papandreou et al. 2011). Targeting cell surface GRP78 in colon and lung cancer in mice with a monoclonal antibody (mAb159) causes tumor regression in vivo (Liu et al. 2013). Lastly, inhibition of GRP78 activation with active compounds present within the herbal medicine Ponciri fructis or the pyrone-type polyketide, verrucosidin, exhibits selective cytotoxicity in human pancreatic cancer cells or colon cancer cells undergoing glucose deprivation-induced ER stress (Park et al. 2007; Cha et al. 2009).

Inducing ER stress while concomitantly inhibiting the adaptive UPR has also begun to find experimental support. For instance, it has been shown that the mechanism of bortezomib's cytotoxic activity in myeloma cells is its ability to inhibit *Xbp1* splicing via stabilization of unspliced XBP-1, which acts as a dominant negative suppressor of XBP1-s, while inducing ER stress via proteasome inhibition (Lee et al. 2003a). Congruently, the induction of ER stress with bortezomib or 17-AAG in myeloma cells was shown to synergize with the activity of transgenic or small molecule-mediated inhibition of *Xbp-1* splicing resulting in the induction of greater and irreparable cytotoxicity than either agent alone *in vitro* and *in vivo* (Lee et al. 2003a; Mimura et al. 2012). In human pancreatic cancer cells, bortezomib reduces GRP78 and CHOP expression under ER stress conditions and sensitizes them to ER stress-inducing compounds, including thapsigargin, tunicamycin, and cisplatin, yielding synergistic cytotoxicity *in vitro* and *in vivo* (Nawrocki et al. 2005). GSK2606414, a small molecule inhibitor of PERK autophophorylation and downstream eIF2α phosphorylation, cooperates with ER stress induced by hypoxia or thapsigargin, causing greater inhibition of *in vitro* clonogenic survival of pancreatic and colon cancer cells than either PERK inhibition or ER stress induction alone (Axten et al. 2012; Cojocari et al. 2013). Epigallocatechin gallate, which inhibits GRP78 by targeting its ATP-binding domain, sensitizes human glioma cells to ER stress induced by the chemotherapeutic agent, temozolomide, resulting in synergistic cyotoxicity, greater than either agent alone (Pyrko et al. 2007). There are several chemical UPR inhibitors that have displayed efficacy against tumor growth *in vitro* and *in vivo* (reviewed in (Li et al. 2011) and (Schonthal 2013)) these are presented in Tables 18.3 and 18.4.

While there has been recent interest in developing UPR inhibitors active against tumor cells, there has been little or no investigation the effect of tumor UPR inhibition on the host anti-tumor immune response. A link between the tumor UPR and the immune response was originally suggested by the finding that silencing of *Grp78* in

Table 18.3 Tumor cell active ER stress/UPR modulators

Compound	Mechanism of action	Tumor cell type	Reference
Thapsigargin, celecoxib	ER stress induction via SERCA inhibition	Breast, prostate	(Denmeade et al. 2012; Johnson et al. 2002; Schonthal 2013)
Biguanides, versipelostatin, pyruvinium pamoate	Inhibition of GRP78, ATF6, Xbp1 response to glucose deprivation	Fibrosarcoma, HeLa	(Saito et al. 2009)
Verrucosidin, Epigallocatechin, *Ponciri fructis* active compound	Inhibition of GRP78	Breast, glioblastoma	(Cha et al. 2009; Park et al. 2007; Pyrko et al. 2007)
STF-083010, MKC-3946	IRE1 endoribonuclease inhibition	Multiple myeloma	(Papandreou et al. 2011); (Mimura et al. 2012)
Irestatin	IRE1 endoribonuclease inhibition	Multiple myeloma, colon	(Papandreou et al. 2011)
Bortezomib	IRE1 dominant negative inhibition	Multiple myeloma	(Lee et al. 2003a; Mimura et al. 2012)
	ER stress induction via proteasome inhibition		
GSK2606414	PERK kinase inhibition	Pancreas, colorectal	(Axten et al. 2012; Cojocari et al. 2013)

Table 18.4 Cell-nonautonomous control of cellular stress responses

Stress response system	Organism	Transmitter (effectors)	Receiver (sensors)	Soluble mediators	Reference
HSR	C. elegans	Thermosensory neuron (ND)	Muscle cell (ND)	ND (unc31-mediated)	(Prahlad and Morimoto 2011)
HSR	C. elegans	Motor neuron (NT)	Muscle cell (postsynaptic NT receptor)	ACh, GABA	(Garcia et al. 2007)
HSR	C. elegans	Muscle, intestinal cell (pha-4)	intestinal, pharyngeal, excretory cells (pha-4)	ND (unc31/unc13-independent)	(van Oosten-Hawle et al. 2013)
UPR^{MT}	C. elegans	Neuron (ND)	Intestinal cell (ND)	ND	(Durieux et al. 2011)
UPR^{ER}	C. elegans	Neuron (ND)	Multiple somatic tissues (ND)	ND	(Sun et al. 2012)
UPR^{ER}	C. elegans	Neuron (xbp1)	Intestinal cell (xbp1)	ND (unc13-mediated)	(Taylor and Dillin 2013)
UPR^{ER}	Mouse	Epithelial tumor cell (IRE1)	Macrophage	ND	(Mahadevan et al. 2012; Mahadevan et al. 2011b; Rodvold et al. 2014a)
	Human		Myeloid dendritic cell		
			Tumor cell (TLR4, IRE1)		

mouse fibrosarcoma cells inhibited growth in an *in vivo* syngeneic transplantation model due, in part, to increased tumor cell-specific memory T cell generation (Jamora et al. 1996). In addition, overexpression of GRP78 in murine insulinoma cells leads to impaired CD8 T cell priming and inhibition of killing, when GRP78-overexpressing tumor cells were used to prime cytotoxic T cell lines, as targets, respectively (Wang et al. 2007). Discovery and characterization of the effect of TERS on host immunity has continued this line of inquiry (Zanetti 2013).

Based on our findings on transmissible ER stress, it appears that a fruitful avenue for therapeutic development will be to develop decoy systems (antibodies, aptamers, etc.) to intercept the TERS factor(s) in the extracellular space (Fig. 18.5a). In this scenario neutralization of TERS would also inhibit the polarization of myeloid cells to a pro-inflammatory/suppressive phenotype, and in turn prevent and unfetter the untoward effects on T cell-mediated immunity, perhaps permitting more effective autochthonous or vaccine-induced anti-tumor immune responses. In addition, TERS may induce tumor-infiltrating myeloid cells to produce tumorigenic cytokines and adversely affects antigen presentation (see Sects. 3.2 and 3.3 for discussion). Lastly, as the downstream effects of TERS on T cell priming are elucidated (i.e. polarization toward a suppressive phenotype), new targets for therapy will come to light (e.g., ecto-ATPases, immune checkpoint molecules, UPR signaling components). Targeting the tumor-infiltrating myeloid cell UPR, the tumor cell UPR, and ultimately suppressive T cells, will reset the multifaceted dysregulation of the tumor microenvironment that hinders anti-tumor immunity.

18.9 Conclusions and Perspectives

During their growth, tumor cells are subjected to *noxae* that exist in the tumor microenvironment and are able to induce the ER stress response that, as discussed, leads to multifaceted dyregulation. Some of them are cell-intrinsic in nature and promote tumor cell adaptation and survival. Others are cell-extrinsic and affect the function of neighboring cells—immune cells, cancer cell themselves (Rodvold et al. 2014a), and stromal cells in a significant way. The intent of this chapter was to bring attention to effects on immune cells.

A hitherto unappreciated phenomenon, transmissible ER stress (TERS), seems to link together tumor cells and immune cells. Receiver myeloid cells, macrophages and dendritic cells become polarized, via ER stress transmission, to a mixed, pro-inflammatory/suppressive phenotype. The pro-inflammatory component, an exquisite innate immune trait, is per se sufficient to fuel tumor growth through a variety of mechanisms including perhaps increasing tumor cell proliferation, survival, and the mutational rate (Grivennikov et al. 2010; Tili et al. 2011). Importantly, however, we found that a mixed, pro-inflammatory/suppressive phenotype also hampers essential immunological functions of dendritic cells, i.e., antigen presentation and T cell priming, hence disabling the adaptive T cell response (Mahadevan and Zanetti 2011; Zanetti 2013). Combined, these effects favor faster tumor growth (Fig. 18.6).

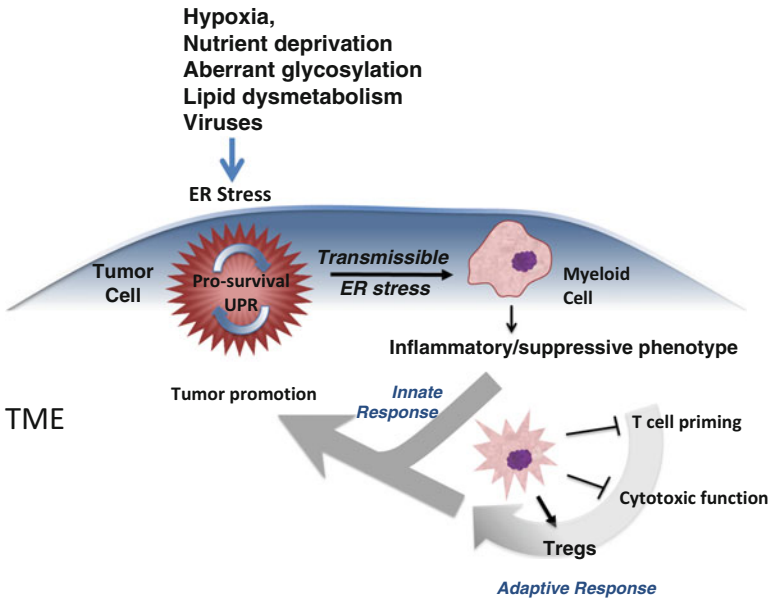

Fig. 18.6 Cell non-autonomous regulation of immunity within the tumor microenvironment via transmissible ER stress. Various *noxae* in the tumor microenvironment (*TME*) perturb tumor cells inducing a UPR. Under the umbrella of the UPR response (*blue shade*) there occur multiple events, which ultimately influence neighboring myeloid cells and T cells. In this framework, the tumor UPR acts as a cell-intrinsic tumor pro-survival factor (*circular arrow*). ER-stressed tumor cells also release many factors (cytokines, chemokines metalloproteinases etc.) among which is a factor(s) able to polarize myeloid cells by transmitting ER stress to them (*second hand stress*). As a result receiver myeloid cells undergo a UPR, and acquire an inflammatory/suppressive phenotype that facilitates tumor growth directly (innate response) and/or via dysregulation of T cell immunity (adaptive response). Together, these T cell-independent and –dependent effects (*merging arrows*) favor tumor growth (Adapted from Mahadevan and Zanetti (2011))

That ER stress can be transmitted from tumor cells to myeloid cells is a new facet in the complex intercellular interplay of the tumor microenvironment. A group of cancer cells (those experiencing a ER stress response) influencing the community of neighboring cells is reminiscent of *quorum sensing* in bacteria (Miller and Bassler 2001). Similar to the growth advantage conferred to bacteria by *quorum sensing*, the transmission of ER stress empowers a group of cancer cells to control the tumor microenvironment and initiate a cascade of pro-tumorigenic events. This new mechanism of immune subversion could explain, at least in part, why autochthonous or vaccine-induced anti-cancer T cell responses are ineffective at controlling tumor growth.

Cell non-autonomous control of several protein-folding homeostatic systems has been recently identified. These include the heat shock response (HSR), the mitochondrial UPR (UPR^{mt}), and the endoplasmic UPR (UPR^{ER}) in the nematode, *Caenorhabditis elegans*. In each of these systems, proteotoxic stress adaptation signaling in one cell (e.g. neuron, muscle cell) is sensed in a distal cell (e.g. intestinal cell), which

upregulates its own proteotoxic stress adaptation machinery, leading to cellular adaptation, resistance to death, and in some cases, organismal longevity. No soluble factors mediating this cell non-autonomous stress signaling have yet been identified, though it is suggested that neuron-derived vesicular trafficking is involved in some, but not all, systems of cell non-autonomous control of proteotoxic stress (reviewed in (Taylor and Dillin 2013)). It is possible that TERS-based tumor-myeloid cell communication is an evolutionary descendant of these more ancient intercellular communication networks, and implies that other cellular stress-based signaling may occur in humans, especially in disease states characterized by pathogenic proteostasis and inflammation (e.g. Type I diabetes and neurodegenerative disease).

In conclusion, the cell extrinsic effects of the tumor UPR represent a new variable in the complex and still poorly-understood interplay between cancer and the immune system. We have presented some general principles for interfering with the UPR within the tumor microenvironment. In our opinion, two important practical considerations can be made. One is that by interfering with the UPR in cancer cells one may succeed in causing cell death and also prevent the initiation of transmissible ER stress. The other is to intercept the transmissible ER stress factor(s) or block the effects of transmissible ER stress on receiver myeloid cells. Overall, controlling the UPR within the tumor microenvironment may represent an opportunity to complement conventional therapies and immunotherapy in the future.

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