Regulation of FOXO Stability and Activity by MDM2 E3 Ligase

By

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of the requirements for the degree of
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Dedication

To my parents, my in-laws, my brother,
my husband, Zhigang and my little pumpkin, caroline.

Without their love, understanding and support, I could
not have done even one piece of it.
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ABSTRACT

Members of the forkhead class O (FOXO) transcription factors are tumor suppressors and key molecules that control aging and lifespan. The stability of mammalian FOXO proteins is controlled by proteasome-mediated degradation but general ubiquitin E3 ligases for FOXO factors remain to be defined. The current studies demonstrate that MDM2 bound to FOXO1 and FOXO3A and promoted their ubiquitination and subsequent degradation, a process apparently dependent on FOXO phosphorylation at PKB sites and on the E3 ligase activity of MDM2. The binding occurred between endogenous proteins and was involved the forkhead box of FOXO1 and the region of MDM2 that controls its cellular localization. MDM2 promoted the ubiquitination of FOXO1 in vitro in a cell free system. Knocking down MDM2 by siRNA caused the accumulation of endogenous FOXO3A protein, and enhanced the expression of FOXO target genes. In addition, MDM2 promoted the transcriptional activity of FOXO in a transient transfection system. In cells stably expressing a temperature sensitive mutant p53, activation of p53, by shifting to permissive temperatures led to MDM2 induction and the degradation of endogenous FOXO3A. These data suggested that MDM2 acts downstream of p53 as an E3 ubiquitin ligase to promote the degradation of mammalian FOXO factors.
INTRODUCTION

1. FOXO Family

1.1. FOX Family

Forkead proteins constitute a family of structurally related transcriptional factors that have been identified in all eukaryotes ranging from yeast to human. Forkhead transcription factors are characterized by the forkhead domain, which is a ~100 amino-acid monomeric DNA binding domain. The three-dimensional structure of forkhead domain consists of two W1 and W2 loops (or wings) and three α helices (Lehmann et al., 2003). Due to its butterfly-like appearance, the domain is also known as “winged-helix” domain.

The first member of the forkhead family was identified in 1989 as a nuclear homeotic gene involved in the embryonic development of flies (*Drosophila melanogaster*). To date, over 100 members of the forkhead family have been identified in species ranging from *Saccharomyces cerevisiae* to humans (Kaestner et al., 2000). The nomenclature of the forkhead transcription factors has recently been revised. These genes, now termed Fox (after Forkhead box), are divided into 17 subclasses (A to Q), according to the amino acid sequence of their conserved forkhead domains ([http://www.biology.pomona.edu/fox.html](http://www.biology.pomona.edu/fox.html)).

Comparative genome analyses have shown that the number of forkhead transcription factors appears to have increased during evolution, with a greater number identified in vertebrates than in invertebrates (Lehmann et al., 2003). The origin and
expansion of forkhead genes is positively correlated with eukaryotic complexity because among the organisms for which genome sequences are available, there is a correlation between anatomical complexity and forkhead gene number: 4 FOX genes in \textit{S. cerevisiae}, 15 in \textit{Caenorhabditis elegans}, 20 in \textit{Drosophila melanogaster} and 43 in humans (Mazet et al., 2003). The human FOX gene family consists of: FOXA1, FOXA2, FOXA3, FOXB1, FOXC1, FOXC2, FOXD, FOXD2, FOXD3, FOXD4, FOXD5 (FOXD4L1), FOXD6 (FOXD4L3), FOXE1, FOXE2, FOXE3, FOXF1, FOXF2, FOXG1 (FOXG1B), FOXH1, FOXI1, FOXJ1, FOXJ2, FOXJ3, FOXK1, FOXK2, FOXL1, FOXL2, FOXM1, FOXN1, FOXN2 (HTLF), FOXN3 (CHES1), FOXN4, FOXN5 (FOXR1), FOXN6 (FOXR2), FOXO1 (FOXO1A, FKHR), FOXO2 (FOXO6), FOXO3 (FOXO3A), FOXO4 (MLLT7), FOXP1, FOXP2, FOXP3, FOXP4, and FOXQ1. Members of FOX subfamilies A-G, I-L and Q were grouped into class-1 FOX proteins, while members of FOX subfamilies H and M-P were grouped into class-2 FOX protein. The presence of a C-terminal basic region within the FOX domain is the common feature of class-1 FOX protein (Katoh and Katoh, 2004b).

FOX transcriptional regulators play various roles during development, from organogenesis to language acquisition. Foxb1 is reported to control development of mammary glands and regions of the central nervous system that regulate the milk ejection reflex in mice (Kloetzli et al., 2001). FOXC1 and FOXC2 (Kume et al., 2000; Kume et al., 1998; Wilm et al., 2004), FOXE1 (Aza-Blanc et al., 1993; Ortiz et al., 1997; Zannini et al., 1997), FOXF1 (Kalinichenko et al., 2001; Kalinichenko et al., 2002), FOXJ1 (Pelletier et al., 1998) and FOXJ2 (Granadino et al., 2000) play important roles in the development of the organs, such as thyroid organogenesis, mesonephros, gonad, liver, gallbladder, lung and intestinal tract. FOXD1 (Hatini et al., 1996), FOXE3 (Blixt et al., 2007; Valleix et al., 2006) and FOXL2 (De Baere et al., 2001; De Baere et al., 2002)
play a critical role in the development of eyes. FOXD3 (Alkhateeb et al., 2005; Guo et al., 2002), FOXD5 (Yu et al., 2002) and FOXJ3 (Landgren and Carlsson, 2004) are involved in neural development. FOXD4 (Minoretti et al., 2007) and FOXP4 (Li et al., 2004b) are related to heart development and differentiation. The FOXG1 (Pauley et al., 2006) and FOXI1 (Hulander et al., 2003) are required for the development of the mammalian inner ear. FoxH1 (FAST) is a transcription factor that mediates signaling by transforming growth factor-β, activin, and nodal. FoxH1−/− embryos failed to orient the anterior-posterior (A-P) axis correctly (Yamamoto et al., 2001). FOXJ1 is required for late stages of ciliogenesis (You et al., 2004b). It is essential for nonrandom determination of left-right asymmetry and development of ciliated cells. FOXN4 is necessary and sufficient for commitment to the amacrine cell fate and is nonredundantly required for the amacrine and horizontal cells (Li et al., 2004a). FOXP2 has been implicated in the acquisition of grammatical skills (Hurst et al., 1990; Lai et al., 2001). FOXP3 is the master regulator in the development and function of regulatory T cells and the selected marker for them (Alvarado-Sanchez et al., 2006; Bennett et al., 2001; Brunkow et al., 2001; Suri-Payer and Fritzsching, 2006). Human FOXN1 (Frank et al., 1999) and FOXQ1 (Hong et al., 2001) function in hair differentiation. FOXN1 is also required for the growth and differentiation of thymic epithelial cells (Balciunaite et al., 2002).

FOX factors are also very important for cell growth, differentiation and tumorigenesis. FOXA1 binds to the promoters of more than 100 genes associated with metabolic processes, the regulation of signaling and the cell cycle (Carlsson and Mahlapuu, 2002; Kaestner, 2000; Tomaru et al., 2003). High expression of FOXA1 (Gao et al., 2003; Lin et al., 2002b), FOXN6 (Katoh and Katoh, 2004d), FOXQ1 (Bieller et al., 2001) and FOXM1 (Nakamura et al., 2004) has been reported in various tumors, including lung, esophageal, breast, prostate and pancreatic cancers. Several FOX
factors are thought of as the candidate tumor suppressor genes. This includes FOXC1 (Zhou et al., 2002), FOXK2 (Li et al., 1992), FOXN5 (Katoh and Katoh, 2004a, c) and FOXO family (Medema et al., 2000). These factors are reported either to be inactive in cancer or to suppress tumor cell growth when overexpressed. FOXM1 is expressed in proliferating cells and becomes silenced in terminally differentiated cells (Korver et al., 1997; Yao et al., 1997; Ye et al., 1997). FOXM1 might be beneficial to patients whose lung endothelial-cell barrier has been damaged (Zhao et al., 2006).

FOX factors also regulate chromatin remodeling and transcription. The FOXA3 acts in chromatin remodeling (Kaestner et al., 1994). FOXK1 is essential for regulating cell cycle progression in myogenic progenitor cells (Hawke et al., 2003). FOXL1 is capable of remodeling chromatin higher-order structure and can cause stable and site-specific changes of chromatin by either creating or removing DNase I hypersensitive sites, resulting in changes the proliferation, differentiation, and positioning of epithelial cells (Kaestner et al., 1997; Yan et al., 2006). FOXN3 binds to Ski-interacting protein (SKIP, NCoA-62), which is a transcriptional co-regulator known to associate with a repressor complex (Scott and Plon, 2005).

It is believed that FOX factors form a network. For example, FOXG1, a class-1 FOXO member, acts as a repressor of transcriptional activity of the FOXO family (Aoki et al., 2004). FOXA and FOXO factors cooperate in complex regulatory networks of the pancreas and liver (Czech, 2003).

1.2. FOXO Family

FOXO factors are members of the O-subclass of the class-2 FOX family. A unique five amino acid (GDSNS) insertion immediately prior to helix H3 within the forkhead domain is characteristic of this subfamily. This insertion is absent in all other
FOX transcription factors (Arden, 2006). The first member of FOXO was identified in *C. elegans* (*Caenorhabditis elegans*) and named DAF-16 and was shown to regulate dauer formation in *C. elegans* (Thomas, 1993).

### 1.2.1. Classification of Human FOXO Factors

In invertebrates there is only one FOXO gene, termed *daf-16* in the worm and *dFOXO* in the fly. In mammals there are four functional FOXO genes, FOXO1, 3A, 4, and 6 (Carter and Brunet, 2007) (Table 1).

The FOXO1 gene is located in chromosome 13q14.1. It is the most abundant FOXO isoform in insulin-responsive tissues such as liver, adipose tissue, pancreatic cells, kidney, spleen and brain (Armoni et al., 2006). Embryos of FOXO1 homozygous mice are smaller and die at embryonic day 10.5 due to several embryonic defects (Nakae et al., 2002), such as incomplete vascular development (Hosaka et al., 2004). Analysis of heterozygote mutant mice indicates that FOXO1 is involved in the function of pancreatic β-cells, hepatic glucose metabolism and adipocyte differentiation (Kitamura et al., 2002; Nakae et al., 2002; Nakae et al., 2003).

The FOXO3A gene is located in chromosome 6q21. It is highly expressed in the heart, spleen, lung, kidney, ovary, adipose tissue and brain (Zhu et al., 2004). FOXO3A knockout mice reveal haematological abnormalities, decreased glucose uptake in glucose tolerance tests (Castrillon et al., 2003) and a distinct ovarian phenotype due to premature follicular activation that leads to oocyte death and subsequent depletion of follicles. It has been suggested that FOXO3A acts as a suppressor of follicular activation (Castrillon et al., 2003; Hosaka et al., 2004). In mature endothelial cells, FOXO1 and FOXO3A are the most abundant FOXO isoforms. Overexpression of constitutively active
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<th><strong>Chromosomal translocation</strong></th>
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<td>FKHR</td>
<td>Fkh1, Foxo1a</td>
<td>13q14.1</td>
<td>t(2;13)(q35;q14) PAX3:FOXO1 and t(1;13)(p35;q14) PAX7:FOXO1</td>
<td>Alveolar rhabdomyosarcomas</td>
<td>Ubiquitous. Highest in He, Sp, Ad, Ki, Br</td>
<td>E10.5 lethality angiogenesis defects</td>
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<tr>
<td>FOX01b</td>
<td>FKHR pseudogene 1 (FKHRP1)</td>
<td></td>
<td>5q35.2-35.3</td>
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<td>FOX03a</td>
<td>FKHRL1, AF6q21, FOXO2</td>
<td>Fkh2, Foxo3a</td>
<td>6q21</td>
<td>t(6;11)(q21;q23) MLL:FOXO3</td>
<td>Secondary acute myeloblastic leukemia</td>
<td>Ubiquitous. Highest in He, Br, Sp, Lu, Ki, Ad, Ov</td>
<td>Female sterility, anemia, glucose uptake defects, overproliferation of helper T cells, increased neutrophil apoptosis</td>
</tr>
<tr>
<td>FOX03b</td>
<td>FKHRL1 pseudogene 1 (FKHRL1p1)</td>
<td></td>
<td>17p11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOX04</td>
<td>AFX, AFX1, MLLT7</td>
<td>Afx, Afs, Foxo4, Mllt7</td>
<td>Xq13.1</td>
<td>t(X;11)(q13;q23) MLL:FOXO4</td>
<td>Acute leukemias</td>
<td>Ubiquitous. Highest in He, Br, Sp, Lu</td>
<td>Viable, no defects reported yet</td>
</tr>
<tr>
<td>FOX05</td>
<td>zFKHR</td>
<td>Fish ortholog of FOXO3a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOX06</td>
<td>FOXO6</td>
<td>Foxo6</td>
<td>1p34.1</td>
<td>None identified</td>
<td></td>
<td>Br, Th, Ki</td>
<td>Not done</td>
</tr>
</tbody>
</table>

He, heart; Sp, spleen; Ad, adipose tissue; Ki, kidney; Br, brain; Lu, lung; Ov, ovaries; Th, thymus

Table 1 FOXO family members in mammals (Greer, Brunet 2005)
FOXO1 or FOXO3A significantly inhibits endothelial cell migration and tube formation in vitro (Potente et al., 2005).

FOXO4 gene is located in chromosome Xq13.1. It is abundant in the lungs, brain, heart and skeletal muscle and kidneys (Nakae et al., 2001). FOXO4 knockout mice show no obvious abnormalities (Hosaka et al., 2004).

FOXO6 gene is located in 1p34.1. Embryonic expression of FOXO6 is seen predominantly in the developing brain where it is expressed in a specific temporal and spatial pattern. In the adult animal, FOXO6 expression persists in the nucleus accumbens, cingulated cortex, parts of the amygdale, and hippocampus. FOXO6 is also expressed in kidney and thymus (Jacobs et al., 2003). The phenotype of FOXO6-deficient animals is not reported, but its restricted expression pattern suggests that it may play a role in embryologic development of the central nervous system (Hoekman et al., 2006).

1.2.2. Domain Structure of FOXO

From the N-terminus to the C-terminus, the FOXO protein contains a proline-rich domain, a forkhead domain, NLS (nuclear localization signal), NES (nuclear export signal), a LxxLL motif and an activation domain (Figure 1). The proline rich motif binds to the CH3 region of CBP/p300 and stabilizes the interaction between FOXO factors and CBP/p300 (van der Heide and Smidt, 2005). The Forkhead domain is responsible for binding to target gene promoters. The central region of FOXO includes the NLS and NES and accounts for the cellular localization of FOXO. The LxxLL motif is reported to bind to SIRT1 and has an important role in regulating its transcriptional activity (Nakae et al., 2006). The C-terminus of FOXO contains the activation domain which stimulates the promoter activity (Yang et al., 2005). In the nucleus, all FOXO proteins bind to DNA as
Figure 1  Structure of human FOXO. Full-length FOXO and known motifs are presented. *PxPxP*-proline rich motif; *FOX*-forkhead domain; *LxxLL*-L, leucine; *X*, any amino acid; *AD*-activation domain; *Nt*-amino terminal; *NLS*-nuclear localization signal; *NES*-nuclear export signal; *Ct*-carboxyl terminal. The *numbers above the drawings* denote amino acid numbers.
monomers through the FOX domain. The core motif of the consensus recognition site for FOXO on DNA is GTAAA (C/T) A and it is designated as the DBE for DAF-16 family member-binding element.

1.3. Functions of FOXO Factors

Studies in *C. elegans* showed that direct activation of DAF-16, or mutation of the insulin-PI3K (phosphotidylinositol 3 kinase)-PKB (protein kinase B) pathway results in extension of lifespan, stress resistance and arrest at the dauer diapause stage (Ogg et al., 1997). Besides cell-autonomous inputs, DAF-16 also responds to environmental cues such as starvation, heat and oxidative stress. All these stress signals activate DAF-16, whereas nutrient-rich conditions deactivate it. dFOXO, the unique FOXO homologue in *Drosophila*, seems to play similar roles, which are negatively controlled by the insulin-PI3K-PKB signaling cascade and nutrients (Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003), although dFOXO-knockout flies are viable and of normal size, they are more vulnerable to the oxidative stress. These data suggest that dFOXO offers protection against oxidative stress.

In humans, the pivotal role of FOXO on cell fate decisions depends on the balance between growth factor stimulation versus cellular stress and damage. Both circumstantial and direct evidences implicate a role of FOXO factors in cancer. The first three members of FOXO were found at chromosomal translocations in tumors. The FOXO1 gene is fused to PAX3 or PAX7 gene in rhabdomyosarcoma (Galili et al., 1993; Sorensen et al., 2002). The FOXO3A gene is fused to MLL gene in secondary acute myeloblastic leukemia (Hillion et al., 1997). The FOXO4 gene is fused to MLL gene in acute lymphocytic leukemia (Parry et al., 1994). Nuclear exclusion of FOXO3A in primary breast tumors correlates with PI3K activation and poor survival of the patients (Hu et al., 2004). FOXO factors reduce tumorigenicity in nude mice (Hu et al., 2004;
Ramaswamy et al., 2002). Furthermore, FOXO proteins interact with many oncogenes such as β-catenin (Essers et al., 2005) or tumor suppressors such as p53 (Brunet et al., 2004).

The physiological importance of FOXO transcription factors is diverse as revealed by loss and gain of function experiments in transgenic and knockout mice. All lines of evidence point to FOXO factors as central regulators of metabolism, aging, proliferation and differentiation (Hosaka et al., 2004). Since a wide range of human diseases, including cancer, have striking aging-dependent onset, FOXO factors are believed to serve as molecular links between longevity and tumor suppression (Greer and Brunet, 2005).

1.3.1. FOXO and Cell Cycle Checkpoint and DNA Repair

Mammalian cells have evolved an intricate defense network to maintain genomic integrity by preventing the fixation of permanent damage from endogenous and exogenous mutagens. Cell cycle checkpoints, a major genomic surveillance mechanism acting at the G1/S and G2/M boundaries, are regulated in response to DNA damage (Hartwell and Weinert, 1989). Defects in these steps may result in a mutated phenotype that is associated with tumorigenesis.

1.3.1.1. FOXO and G1/S Checkpoint

p21 is a cyclin-dependent kinase inhibitor. Loss of p21 expression has been described as a poor prognostic factor and as an independent predictor of bladder cancer progression in muscle invasive cancer (Stein et al., 1998). p27 is a protein that binds to cyclins and cdk to block the entry into S phase. Loss of p27 expression has been shown to be associated with aggressive behavior in a variety of human epithelial tumors, including prostate (Macri and Loda, 1998) and breast (Catzavelos et al., 1997; Gillett et
al., 1999; Tan et al., 1997; Wu et al., 1999) cancers. p130 belongs to the RB family. A study in mice showed that p130 can induce cell cycle arrest (Classon et al., 2000). Mammalian cyclins are classified into 12 different types, from cyclins A to I, based on structural similarity, function period in the cell division cycle, and regulated protein expression. cyclin D1 is a key regulator of the G1 phase of the cell division cycle where it binds to and activates the cyclin-dependent kinases CDK4 and CDK6. In the cells that have the capacity to divide, the main effect of the expression of active forms of FOXO family members is to promote cell cycle arrest at the G1/S boundary. FOXO factors play a major role in G1 arrest by upregulating cell cycle inhibitors such as p21 (Hauck et al., 2007; Lawlor and Rotwein, 2000), p27 (Medema et al., 2000; Stahl et al., 2002) and p130 (Chen et al., 2006) and by repressing cell cycles activators cyclin D1 and D2 (Schmidt et al., 2002).

1.3.1.2. FOXO and G2/M Checkpoint and DNA Repair

GADD45 is growth arrest and DNA damage-inducible protein 45. It was identified on the basis of its rapid transcriptional induction after UV irradiation (Fornace et al., 1989) and can induce G2/M arrest (Wang et al., 1999). Induction of GADD45 is also observed after several types of pathological stimuli including various environmental stresses: hypoxia, irradiation, genotoxic drug exposure and growth-factor withdrawal (Papathanasiou et al., 1991). Three G-type cyclins have been identified: cyclins G1, G2, and I. All three are expressed in terminally differentiated cells, act as cell cycle inhibitors in certain cell types and may induce cell cycle arrest (Martinez-Gac et al., 2004). Microarray analysis led to the identification of GADD45 and cyclin G2 as the downstream target genes of FOXO3A for G2/M arrest and DNA repair (Tran et al., 2002).
In vivo experiment showed that FOXO3A and FOXO4 activate GADD45 promoter through direct interaction with the DBE. Oxidative stress activates the GADD45 promoter in a FOXO-dependent manner, resulting in increased level of GADD45 mRNA and protein as well as G2 arrest (Furukawa-Hibi et al., 2002). In response to glucose starvation, FOXO1 also affects GADD45 mRNA level in islets (Martinez et al., 2006).

Cyclin G2 is expressed in various amounts during the cell cycle in lymphocytes (Horne et al., 1997; Horne et al., 1996). Ectopic expression of cyclin G2 inhibits cell cycle progression (Bennin et al., 2002). FOXO3A interacts with cyclin G2 in mouse fibroblasts. Active forms of FOXO3A increase cyclin G2 mRNA levels by activating the cyclin G2 transactivation through interaction with the DBE in its promoter (Martinez-Gac et al., 2004).

One mechanism by which cells protect themselves against stress is by repairing the damage to their DNA and proteins that occurs upon exposure to environmental stress. Furthermore, this capacity to repair DNA damage is closely correlated with an increased longevity in mammals (Kirkwood and Austad, 2000). Gene array analysis showed that FOXO3A is involved in nuclear excision DNA repair by modulating the expression of GADD45 and DDB (damaged DNA binding) protein (Tran et al., 2002).

1.3.2. FOXO and Apoptosis

FOXO factors have been shown to mediate apoptosis by activating proapoptotic genes in a variety of cells. FOXO promotes cell death through these downstream targets: TRAIL (Tumor necrosis-related apoptosis-inducing ligand), FasL (Fas Ligand) (Brunet et al., 1999), Bim (Stahl et al., 2002; Sunters et al., 2003; Urbich et al., 2005) and PUMA (You et al., 2006a).

TRAIL is a member of the TNF family of cytokines. It induces apoptosis via death receptors (DR4 and DR5) in a wide variety of tumor cells but not in normal cells (Suliman
et al., 2001). TRAIL is a direct target of FOXO3A. FOXO3A response element is located in TRAIL promoter spanning nucleotides -138 to -121. Decreased activity of FOXO3A and FOXO1 in prostate cancers resulting from loss of PTEN leads to a decrease in TRAIL expression that can contribute to increased survival of the tumor cells (Modur et al., 2002).

FasL is a type II transmembrane protein. It functions as a homotrimer, because it trimerizes Fas receptor, which spans the membrane of the "target" cell. This trimerization usually leads to apoptosis. There are three putative FOXO binding elements in the FasL promoter. In the human leukemia T cell line Jurkat, FOXO3A induces apoptosis by activating FasL expression and the Fas-FasL apoptotic pathway (Yamamura et al., 2006).

Bim is a BH3-only Bcl2 family member. Bim (also known as Bcl2l11) provokes apoptosis (O'Connor et al., 1998). There are two conserved FOXO binding sites in the Bim promoter: one at position -204 relative to the transcription start site (bim1) and one at the boundary between exon 1 and the first intro. FOXO3A directly activates the bim promoter via the two conserved FOXO binding sites (Gilley et al., 2003).

PUMA is an essential mediator of p53-dependent and -independent apoptosis in vivo. Promoter analysis identifies that there is a potential consensus FOXO-responsive element in intron 1 of PUMA which is conserved in humans and mice. FOXO3A up-regulates PUMA transcription in response to cytokine or growth factor deprivation (You et al., 2006a).

1.3.3. FOXO and Atrophy

Atrophy is the partial or complete decompositon of a part of the body. Causes of atrophy include poor nourishment, poor circulation, loss of hormonal support, loss of nerve supply to the target organ, disuse or lack of exercise or disease intrinsic to the
tissue itself. Hormone and nerve inputs that maintain an organ or body part are referred to as trophic. Atrophy is a general physiological process of reabsorption and breakdown of tissues, involving apoptosis on a cellular level. When it occurs as a result of disease or loss of trophic support due to other disease, it is termed pathological atrophy, although it can be a part of normal body development and homeostasis as well.

Skeletal muscle atrophy is a debilitating response to fasting, disuse, cancer, and other systemic diseases. FOXO3A acts on the atrogin-1 promoter to cause atrogin-1 transcription and dramatic atrophy of myotubes and muscle fibers. When FOXO activation is blocked by a dominant-negative construct in myotubes or by RNAi in mouse muscles in vivo, atrogin-1 induction is prevented during starvation and atrophy of myotubes induced by glucocorticoids (Sandri et al., 2004).

In fully differentiated skeletal and cardiac muscle cells, expression of a constitutively active form of FOXO3A causes atrophy. The atrophy is not due to apoptosis. Instead, it is due to a decrease in cell size.

1.3.4. FOXO and ROS Detoxification in Stem Cells

FOXO proteins have been reported to allow detoxification of ROS (reactive oxygen species) by upregulating free radical scavenging enzymes, including MnSOD (manganese superoxide dismutase) and catalase (Storz, 2006).

FOXO3A protects quiescent cells from oxidative stress by directly increasing MnSOD mRNA and protein levels. This increase in protection from reactive oxygen species antagonizes the apoptosis caused by glucose deprivation. Increased resistance to oxidative stress is also associated with longevity. The model of Forkhead involvement in regulating longevity stems from genetic analysis in C. elegans, but the model also can be extendable to mammalian systems (Kops et al., 2002).
Stem cells are primal cells found in all multi-cellular organisms. They have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second characteristic is that under certain physiologic or experimental conditions they can be induced to become cells with special functions such as the beating cells of the heart or the insulin-producing cells of the pancreas. Regulation of oxidative stress in the HSC (hematopoietic stem cell) compartment is critical for the maintenance of HSC self-renewal. FOXO-dependent signaling is required for the long-term regenerative potential of the HSC compartment through regulation of HSC responses to physiologic oxidative stress, quiescence and survival. Further analysis of ROS levels in FOXO knockout mice showed that FOXOs affect HSC integrity by regulating ROS. Myeloid progenitor cells isolated from FOXO1/3/4 conditional knockout animals show decreased ROS (Tothova et al., 2007).

1.3.5. FOXO and Tissue Differentiation

Cell and tissue differentiation process systematically bases on a number of gene expressions that commence successively along with the passage of time. In differentiating cells, FOXO factors have been implicated in inhibiting and promoting differentiation, depending on the cell types and different FOXO isoforms. In adipocytes, FOXO1 is induced in the early stages of adipocyte differentiation but its activation is delayed until the end of the clonal expansion phase. Constitutively active FOXO1 prevents the differentiation of preadipocytes, while dominant-negative FOXO1 restores adipocyte differentiation of fibroblasts from insulin receptor-deficient mice. FOXO1 directly inhibits the differentiation through upregulation of p21 (Nakae et al., 2003).

Hematopoiesis, or blood cell formation, is the process in which a limited set of hematopoietic stem cells is able to give rise to all types of functional blood cells via
commitment to specific hematopoietic lineages. FOXO3A can promote erythroid differentiation through BTG1 (B-cell translocation gene 1). BTG1, in turn, modulates arginine methylation necessary for erythroid differentiation (Bakker et al., 2004). Furthermore, recent experiments demonstrated that FOXO3A directly binds and represses the transcription of the ID1 (inhibitor of differentiation 1), a suppressor of erythroid differentiation, through the recruitment of an HDAC1 (histone deacetylase 1)–mSin3a complex (Lam et al., 2006).

1.3.6. FOXO and Glucose and Energy Metabolism

FOXO factors play an important role in upregulating genes that control glucose metabolism. They upregulate G6Pase (glucose-6-phosphatase) which is responsible for converting glucose-6-phosphate to glucose (Nakae et al., 2001; Onuma et al., 2006) and PEPCK (phosphoenolpyruvate carboxykinase) which converts oxaloacetate to phosphoenolpyruvate (Samuel et al., 2006). In the absence of insulin, FOXO1 binds to IRE (insulin response elements) in G6Pase and PEPCK promoters and stimulates their promoter activity. However, in the presence of insulin, FOXO1 undergoes phosphorylation and is exported out of the nucleus, ceasing its transcriptional activity (Kitamura et al., 2002). In liver and muscle, FOXO proteins can stimulate PDK4 (pyruvate dehydrogenase kinase-4) expression, which limits oxidative metabolism of glucose and conserves glucose for utilization in other tissues (Furuyama et al., 2003).

FOXOs are also involved in lipid metabolism through the regulation of AdipoR1/2 (Tsuchida et al., 2004), LPL (lipoprotein lipase) (Kamei et al., 2003), HMGCS2 (mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase) (Nadal et al., 2002) and SCP (sterol carrier protein) (Dansen et al., 2004) gene expression. Adiponectin/Acrp30 is a hormone secreted by adipocytes, which acts as an antidiabetic and antiatherogenic adipokine. AdipoR1 and -R2 serve as the receptors for adiponectin and mediate
increased fatty acid oxidation and glucose uptake by adiponectin. Mouse study shows that insulin reduces the expression of AdipoR1/R2 via the PI3K/FOXO1-dependent pathway \textit{in vitro} (Tothova et al., 2007). FOXO proteins also stimulate the expression of both PDK4 (Furuyama et al., 2003; Kwon et al., 2004), which limits the flux of pyruvate through the Krebs cycle, and the expression of LPL (Kamei et al., 2003). HMGCS2 is a key enzyme controlling ketogenesis. Deletion analysis showed that there is a FOXO3A-responsive sequence AAAAATA located 211 bp upstream of the HMGCS2 gene transcription start site. FOXO3A can stimulate transcription of HMGCS2 and this stimulation is repressed by insulin (Nadal et al., 2002). The SCP (sterol carrier protein) gene encodes two proteins, SCPx and SCP2, which are independently regulated by separate promoters. SCPx has been shown to be the thiolase involved in the breakdown of branched-chain fatty acids and in the biosynthesis of bile acids. Both SCPx and SCP2 are upregulated by FOXO3A (Dansen et al., 2004).

1.3.7. FOXO and Longevity

A number of diseases, including cancer, type 2 diabetes and neurodegenerative disorders have a striking age-dependent onset. A number of studies now support the hypothesis that longevity could be affected by simple changes in the environment. Organismal longevity also has a genetic component. A pivotal breakthrough in identifying genes involved in longevity came from studies in \textit{C. elegans}. Some worm mutants can live two to three times longer than wild type worms (Kenyon et al., 1993). These long-living worms turned out to have mutations in the insulin receptor gene (Kimura et al., 1997). Remarkably, flies and mice that have mutations in the insulin or the IGF-1 (insulin like growth factor-1) receptor genes are also long-lived (Bluher et al., 2003; Holzenberger et al., 2003; Tatar et al., 2001). These results indicate that insulin and IGF-1 regulate longevity in a conserved manner throughout species.
A link between FOXO factors and aging was also initially observed in invertebrates. In *C. elegans*, DAF-16 regulates lifespan (Baumeister et al., 2006). In *Drosophila melanogaster*, dFOXO regulates age-linked decline of cardiac function and longevity (Giannakou et al., 2004; Giannakou and Partridge, 2004; Hwangbo et al., 2004; Wessells et al., 2004).

But so far, there is no direct evidence to link FOXO factors and longevity in mammals. Since FOXO factors are involved in cellular resistance to stress and promote ROS detoxification, DNA repair, and cell cycle arrest to allow time for the repair process, they are projected to reduce detrimental effects of aging in mammals (Glauser and Schlegel, 2007). Thus, a role of FOXO factors in longevity of mammals is expected.

### 1.4. Posttranslational Modifications of FOXO Proteins

As described above, FOXO proteins have diverse cellular functions by acting as transcription factors. The activity of FOXO proteins can be regulated by posttranslational modifications including phosphorylation, acetylation and ubiquitination.

#### 1.4.1. Phosphorylation of FOXO

Phosphorylation is a process wherein phosphate groups are added to proteins by protein kinases or removed from proteins by protein phosphatases. The addition or removal of phosphate groups dramatically alters protein function and leads to a myriad of biological responses. Phosphorylation is shown to play a major role in the regulation of FOXO cellular localization, transcription activity and protein stability, and is mediated mainly by PKB.

#### 1.4.1.1. PKB and FOXO

PKB is a serine/threonine kinase. It plays a pivotal role in cell survival and proliferation through a number of downstream effectors. All FOXO family members,
except FOXO6, contain three PKB phosphorylation sites (RXRXX(S/T)X). DAF-16, the *C. elegans* FOXO, contains four putative PKB recognition motifs. Two are located at end of the N-terminal & C-terminal of DAF-16. The other two sites are located in the forkhead domain and overlap. Disrupting PKB-consensus phosphorylation sites in DAF-16 causes nuclear accumulation in wild-type animals but has little effect on lifespan (Lin et al., 2001; Van Der Heide et al., 2004).

PKB is shown to phosphorylate human FOXO family members on the three PKB motifs: an N-terminal threonine (Thr32), a forkhead box serine (Ser253), and a more C-terminal serine (Ser315). Thr32 is preferentially phosphorylated by PKB. This phosphorylation is shown to inhibit p300 binding to FOXO3A, resulting in the recruitment of RanGTPase and CRM1 protein to transport FOXO3A to cytoplasm and to bind the 14-3-3 chaperone (Mahmud et al., 2002). Mutation of Ser253 to a neutral residue, such as alanine (S253A), inhibits further phosphorylation of FOXO3A in other regions of the proteins by PKB and other kinases. This suggests that this site is necessary to prime other sites for phosphorylation. The FOXO3A S253A mutant is constitutively nuclear under different conditions including PKB activation. Therefore, the S253 is called the “gatekeeper site”. This site is believed to inhibit DNA binding, to change the conformation and to inhibit nuclear re-import (Brownawell et al., 2001; Brunet et al., 1999; Brunet et al., 2001; Woods and Rena, 2002). Phosphorylation of Ser315 is shown to enhance nuclear export (Brunet et al., 2001; Rena et al., 2002). All FOXO proteins have been shown to require the consensus N-terminal PKB site and the PKB site located in the forkhead domain in order to translocate from nucleus to cytosol (Brownawell et al., 2001).

Interestingly, the regulation of FOXO6 is different from those of FOXO1, FOXO3A and FOXO4. FOXO6 only contains two PKB/SGK regulatory phosphorylation
sites (Thr26 and Ser184 in mouse FOXO6) (Jacobs et al., 2003). Unlike the other FOXO isoforms, FOXO6 is mostly nuclear. However, FOXO6 phosphorylation at Thr26 and Ser184 appears to decrease its transcriptional activity (van der Heide and Smidt, 2005), which is similar to the effect of PKB on other FOXO factors transcriptional activity.

1.4.1.2. SGK and FOXO

SGKs (serum- and glucocorticoid-induced kinases) belong to a new family of serine/threonine kinases that are regulated at both the transcriptional and posttranslational levels by external stimulation. SGK mediates the biological effects of PI3K in parallel with PKB. Although SGK is closely related to PKB, SGK and PKB display unique features. First, SGK protein expression is induced by extracellular stimuli, but PKB might not be. Second, SGK does not have a pleckstrin homology domain and appears not to be recruited to the plasma membrane prior to its activation. Third, the consensus sequence that is phosphorylated by SGK is not identical to the site phosphorylated by PKB. Fourth, SGK, in contrast to PKB, is capable of phosphorylating peptide substrates that do not have a bulky hydrophobic amino acid immediately C-terminal to the phosphate-acceptor site (Kobayashi and Cohen, 1999).

Activated SGK promotes cell survival in part by phosphorylating and inactivating FOXO3A. In the presence of DNA damage, caused by UV and irradiation treatment, SGK is induced in a p53-dependent manner, leading to the phosphorylation of FOXO3A (You et al., 2004a). SGK, like PKB, phosphorylates FOXO3A, thereby causing FOXO3A translocation from the nucleus to the cytoplasm and inhibition of FOXO3A-dependent transcription. However, SGK and PKB, when expressed at physiological levels, display differences with respect to the efficacy with which they phosphorylate the regulatory sites on FOXO3A. Specifically, Thr32 is phosphorylated by both protein kinases, but SGK prefers Ser315 whereas PKB favors Ser253 (Brunet et al., 2001).
1.4.1.3. IκB Kinase and FOXO3A

The IKK (IκB kinase) signaling pathway is a key survival and antiapoptotic mechanism, aberrant expression of IKK has been implicated in constitutive activation of NF-κB in human breast cancer cell lines and primary tumors. In breast cancer, IKK physically interacts with, phosphorylates, and inhibits FOXO3A independently of PKB. Cytoplasmic FOXO3A correlates with expression of IκKβ or phospho-PKB in many tumors and associates with poor survival in breast cancer (Hu et al., 2004).

1.4.1.4. CK1 and FOXO

CK1 (Casein Kinase 1) is a serine/threonine protein kinase (Hathaway and Traugh, 1979) that phosphorylates FOXO1 at Ser322 and Ser325 following the phosphorylation of Ser319 by PKB or SGK. Multi-site phosphorylation of the region containing Ser319, Ser322, Ser325 and Ser329 provides a signal for the nuclear exclusion of FOXO1 (Rena et al., 2002).

1.4.1.5. CDK2 and FOXO

CDK2 phosphorylates FOXO1 at serine-249 (Ser249) in vitro and in vivo. Phosphorylation of FOXO1 at Ser249 results in cytoplasmic localization of FOXO1 and the inhibition of its transcriptional activity. This phosphorylation was abrogated upon DNA damage through the cell cycle checkpoint pathway and was dependent on the protein kinases Chk1 and Chk2 (Huang et al., 2006). Functional interaction between CDK2 and FOXO1 provides a mechanism that regulates apoptotic cell death after double strand DNA breakage.

1.4.1.6. JNK and FOXO

Besides IIS (insulin/IGF signaling) pathway, lifespan can also be increased by activating the stress-responsive JNK (Jun-N-terminal kinase) pathway. In Drosophila,
JNK requires FOXO to extend lifespan. JNK antagonizes IIS, causes nuclear localization of FOXO and induces the expression of FOXO targets including the growth control and stress defense genes. JNK and FOXO also restrict IIS activity systemically by repressing IIS ligand expression in neuroendocrine cells (Wang et al., 2005).

In humans, an increase in ROS levels induces activation of the small GTPase Ral, which in turn leads to the phosphorylation and activation of the stress kinase JNK. Active JNK induces the phosphorylation of Thr447 and Thr451 on FOXO4. Phosphorylation of these residues is essential for FOXO4 transcriptional activity. Consistent with this, H₂O₂ treatment increases FOXO transcriptional activity and translocation of FOXO4 from the cytoplasm to the nucleus and activation of the transcription factor. Activation of FOXO4 through Thr447/451 phosphorylation can induce transcription of MnSOD and catalase, leading to a decrease in ROS levels. Thus, activation of FOXO4 by oxidative stress is part of a negative feedback loop to reduce the levels of oxidative stress in a cell and to prevent damage to DNA, lipids and proteins (Essers et al., 2004).

1.4.1.7. MST1 and FOXO3A

MST1 is a serine/threonine protein kinase that mediates cell death induced by oxidative stress in primary mammalian neurons through direct activation of FOXO transcription factors. MST1 phosphorylates FOXO proteins at a conserved site within the FOX domain (Ser207) disrupting their interaction with 14-3-3 proteins, promoting FOXO nuclear translocation, and thereby inducing cell death in neurons under stress conditions such as hydrogen peroxide treatment. Knockdown of the C. elegans MST1 ortholog CST-1 shortens lifespan and accelerates tissue aging, while overexpression of cst-1 promotes lifespan and delays aging. The cst-1-induced lifespan extension requires daf-16 (Lehtinen et al., 2006).
1.4.2. Acetylation and Deacetylation of FOXO

1.4.2.1. Acetylation and Deacetylation

Protein acetylation is a post-translational modification that transfers an acetyl group to lysine residues. The best example is histone acetylation, which “opens” chromatin’s structure and activates transcription. Deacetylation, on the other hand, induces closed chromatin structure and repression of gene expression (Cohen and Yao, 2004). Protein acetylation is catalyzed by a group of acetyltransferases with different specificities and target consensus sites. CBP/p300 (calcium response element-binding (CREB)-binding protein), PCAF (p300/CBP-associated factor), members of p160 family, Tip60, and TAFII250 are the main acetyltransferases involved in histone regulation and transcription factor acetylation (http://www.chromatin.us/hatdesc.html). Proteins acetylation is reversed by HDACs (histone deacetylases). There are three classes of HDACs. Class I includes HDAC1, 2, 3, and 8. Class II includes HDAC4, 5, 6, 7, 9 and 10 which show high homology to HAD1 yeast deacetylase. There is one HDAC (HDAC11) that shares homology with both class I and II HDACs. Class III HDACs (SIRTs) have an absolute requirement for NAD in vivo and in vitro (de Ruijter et al., 2003; Imai et al., 2000).

1.4.2.2. Acetylation of FOXO

1.4.2.2.1. P300 and CBP

p300 and CBP are large nuclear proteins encoded by two distinct genes. p300/CBP have been implicated in numerous disease processes, including several forms of cancer, cardiac hypertrophy and Huntington’s disease (Bandyopadhyay et al., 2002; Borrow et al., 1996; Deguchi et al., 2003; Gayther et al., 2000; Gusterson et al., 2003; Muraoka et al., 1996; Ross et al., 2002).
The relative low abundance of p300 is rate-limiting in coactivation and corepression of many transcription factors. Thus, p300 serves to integrate diverse signaling pathways involved in metabolism, cellular differentiation and p53-mediated apoptosis processes (Yao et al., 1998). Orchestration of these activities by p300 involves both a scaffolding function to tether transcription factors to target promoters and its enzymatic activity as a HAT (histone acetyl transferase) (Bannister and Kouzarides, 1996; Ogryzko et al., 1996).

In addition to histones, several other non-histone proteins including transcription factors are also acetylated by p300 (Goodman and Smolik, 2000), including p53, estrogen receptor (Wang et al., 2001) and MyoD (Polesskaya et al., 2000). The activity of p300 itself is also subjected to regulation via a number of post-translational modification including phosphorylation, methylation, sumoylation and acetylation (Banerjee et al., 1994; Chevillard-Briet et al., 2002; Girdwood et al., 2003; Thompson et al., 2004; Yaciuk and Moran, 1991; Yadav et al., 2003).

In terms of differentiation, p300 and CBP appear to have numerous functions. There is embryonic lethality of mice nullizygous for p300 (with defects in neurulation and heart development) as well as in mice double heterozygous for p300 and CBP. Animals which are nullizygous for p300 died between days 9 and 11.5 of gestation and showed defects in neurulation, cell proliferation and heart development. Cells derived from p300-deficient embryos displayed specific transcriptional defects and proliferated poorly. Surprisingly, p300 heterozygotes also manifested considerable embryonic lethality. Moreover, double heterozygosity for p300 and CBP was invariably associated with embryonic death. Thus, mouse development is exquisitely sensitive to the overall gene dosage of p300 and CBP (Yao et al., 1998). Further study in the mouse oocytes within primordial follicles finds that p300 and CBP enter into the oocyte nucleus at different
stages of oocyte growth. From the two-cell stage to the blastocyst stage in the pre-implantation mouse embryos, the localizations of p300 and CBP are different, which provide the information that p300 and CBP have different functions in early mouse embryogenesis (Kwok et al., 2006).

1.4.2.2.2. P300/CBP and FOXO Acetylation

Using immunoprecipitation, an interaction between FOXO1, FOXO4, and FOXO3A and CBP as well as between FOXO3A and p300 was found \textit{in vivo} and \textit{in vitro} (Brunet et al., 2004; Daitoku et al., 2004; Fukuoka et al., 2003; Motta et al., 2004; van der Horst et al., 2004).

CBP and p300 play different roles in the regulation of FOXO1 and FOXO4. Mutational analysis show that FOXO1 and FOXO4 interact with CBP via their C-terminal domain, whereas the N-terminal CH1 domain of CBP is required to bind FOXO1 and FOXO4. CBP binds and acetylates FOXO1 at the K (lysine) 242, K245, and K262 residues. Acetylation at these residues by CBP attenuates FOXO1’s ability to bind cognate DNA sequence and promotes the PKB-dependent phosphorylation of FOXO1 (Matsuzaki et al., 2005). CBP interacts with FOXO4 and acetylates it at K186, K189, and K408. Acetylation by CBP inhibited the transcriptional activity of FOXO4 (Daitoku et al., 2004; Fukuoka et al., 2003; van der Horst et al., 2004). However, in transient transfection experiments acetylation by p300 in the C-terminal region of FOXO1 showed that it potently stimulates FOXO1-induced transcription of IGF-binding protein-1. The intrinsic acetyltransferase activity of p300 is required for both activities (Perrot and Rechler, 2005).

As for FOXO3A, p300 and CBP can bind and acetylate it both \textit{in vivo} and \textit{in vitro}, but two different studies show some discrepancies. One study shows that FOXO3A binds p300/CBP at the N-terminus masking the first 52 amino acids at the N-terminus.
under serum starvation conditions (Mahmud et al., 2002). Another study shows that
CBP/p300 binds the C-terminus and forkhead domains of FOXO3A, leading to
acetylation at lysine residues K242, K259, K271, K290, and K569 (Brunet et al., 2004;
Mahmud et al., 2002). Overall, acetylation by p300 stimulates the transcriptional activity
of FOXO3A (Motta et al., 2004).

1.4.2.3. Deacetylation of FOXO by HDAC

1.4.2.3.1. SIRT1
The SIRT1 gene is located in chromosome 10q21.3. It contains 9 exons (NC_000010). The 4,107 bp human Sirt1 mRNA has an open reading frame of 2,244 bp
and encodes a 747 aa protein with a predictive molecular weight of 81.7 kDa (kilodalton)
(Voelter-Mahlknecht and Mahlknecht, 2006). It belongs to the family of mammalian
sirtuins. The founding member of the sirtuin protein family was the silent information
regulator 2 (Sir2) of S. cervisiae (Saccharomyces cerevisiae). Sirtuin proteins are highly
conserved from yeast to humans. In yeast, worms and flies, expression of Sir2 extends
longevity (Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and
Guarente, 2001; Wood et al., 2004). In S. cervisiae, there are five sirtuin homologs,
Hst1-4 and Sir2. In mammals, there are seven sirtuins, SIRT1-7. SIRT1 is the closest
homolog of Sir2, plays important roles in diverse cellular processes including
transcriptional silencing, rDNA recombination, glucose metabolism and energy
homeostasis, DNA repair and cell survival. Due to its dependency on NAD⁺, the activity
of SIRT1 is regulated by NAD⁺/NADH ratio and thus sensitive to the redox status and
cellular metabolism. Similar to Sir2, SIRT1 is potentially a nutrient sensor that regulates
the lifespan of mammals in response to caloric restricition or nutrient starvation.
Several compounds have been shown to inhibit or activate the deacetylase activity of the Sir2 family. One inhibitor, splitomicin, is a cell-permeable lactone derived from β-naphthol; it inhibits the NAD⁺-dependent deacetylase activity of Sir2 \textit{in vitro} (Bedalov et al., 2001). Another inhibitor, nicotinamide, a product of the Sir2 deacetylation reaction, is an inhibitor of Sir2 activity both \textit{in vivo} and \textit{in vitro}. Nicotinamide has been shown to inhibit a Sir2 homolog, SIRT1, a negative p53 regulator, promoting p53-dependent apoptosis in mammalian cells (Luo et al., 2001; Vaziri et al., 2001). The most potent activator was resveratrol, a polyphenol found in red wine, which is implicated in a number of health benefits. In human cells, treatment with a low concentration of resveratrol increased cell survival following DNA damage. Moreover, low resveratrol concentrations decreased the acetylation of p53 at lysine residue 382, a known SIRT1 substrate; however, high resveratrol concentrations caused the opposite (Howitz et al., 2003).

By deacetylating and inactivating the substrates such as p53 (Langley et al., 2002; Vaziri et al., 2001), NFκB (nuclear factor-κB), FOXO (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004), Ku70 (Jeong et al., 2007), PPARγ (Peroxisome proliferator-activated receptor γ) (Picard et al., 2004), PGC-1α (Balaban et al., 2005), AceCS2 (acetyl-CoA synthetase 2), p300 (Bouras et al., 2005), α-tubulin, and HES1 and HEY2 (basic helix–loop–helix transcriptional repressors) (Takata and Ishikawa, 2003), SIRT1 plays important roles in regulating various cellular processes, including stress response (Vaziri et al., 2001), embryogenesis (McBurney et al., 2003), metabolism (Leibiger and Berggren, 2006), calorie restriction (Brunet et al., 2004), neuronal cell survival (Anekonda and Reddy, 2006; Bordone et al., 2006), insulin secretion (Bordone et al., 2006) and aging (Chua et al., 2005).
1.4.2.3.2. **SIRT1 and FOXO Deacetylation**

In mammalian cells, SIRT1 interacts with all the FOXO factors. Co-immunoprecipitation experiments showed that SIRT1 interacts with FOXO1 *in vivo*. Mutation analysis showed that the LxxLL motif in FOXO1 (amino acids 459-463) is critical for the interaction with SIRT1. Mutagenesis of the LxxLL motif eliminates FOXO1 interaction with SIRT1, sustains the acetylated state of FOXO1 and makes FOXO1 insensitive to nicotinamide and resveratrol (Nakae et al., 2006). Yeast two hybridization experiment showed that FHL2 (Four and a half LIM 2), which interacts with the N-terminal of FOXO1 *in vivo* and *in vitro*, can enhance the interaction of FOXO1 and SIRT1 (Yang et al., 2005).

Mammalian SIRT1 has dual effects on FOXO3A and FOXO4. SIRT1 deacetylates FOXO3A, attenuating FOXO-induced apoptosis but potentiates FOXO-induced cell-cycle arrest (Motta et al., 2004). SIRT1 and FOXO3A form a complex in cells in response to oxidative stress, and SIRT1 deacetylates FOXO3A in vitro and in intact cells. The sites of FOXO3A that appear to be primarily deacetylated by SIRT1 are K242, K245, and K262 (Daitoku et al., 2004). The SIRT1 and FOXO3A interaction requires PKB phosphorylation of FOXO3A because constitutive FOXO3A, in which three PKB phosphorylation sites were mutated to alanine, failed to bind SIRT1 under any stress condition (Brunet et al., 2004; Motta et al., 2004). SIRT1 has a dual effect on FOXO3A function: It increases FOXO3A's ability to induce cell cycle arrest and resistance to oxidative stress, but inhibits FOXO3A's ability to induce cell death (Brunet et al., 2004).

In the case of FOXO4, acetylation by CBP inhibits its transcriptional activity. SIRT1-mediated deacetylation precludes FOXO4 inhibition through acetylation and thereby prolongs FOXO4-dependent transcription of stress-regulating genes. A FOXO4 study revealed a molecular mechanism whereby SIRT1 can promote cellular...
Figure 2 The regulation of FOXO. Full-length FOXO and known phosphorylation sites and acetylation sites are presented. K-lysine; T-threonine; S-serine; T-tyrosine; CDK2-cyclin-dependent kinase 2; PKB- protein Kinase B; SGK- serum and glucocorticoid responsive kinase; CK1- casine kinase 1; DYRK- dual-specificity YAK-1-related kinase; JNK- c-Jun N-terminal kinase; IKKβ- IκB kinase β. The numbers above the drawings denote amino acid numbers. Full-length Mdm2 and known motifs are also shown.
survival and increase lifespan (van der Horst et al., 2004), a similar Sir2 function as observed in worms.

1.4.3. Ubiquitination and Deubiquitination of FOXO

1.4.3.1. Ubiquitination and Degradation of FOXO

The degradation of FOXO transcription factors is mediated by the ubiquitin-proteasome pathway. In the presence of insulin and other growth factors, FOXO proteins are relocalized from the nucleus to the cytoplasm and to be degraded via the ubiquitin-proteasome pathway. PKB activation is necessary for insulin promoted ubiquitin-mediated degradation of FOXO1 and FOXO3A via the proteasome pathway (Aoki et al., 2004; Plas and Thompson, 2003).

As for insulin induced proteasomal degradation of FOXO1, insulin treatment decreases endogenous FOXO1 proteins in HepG2 cells and this decrease is suppressed by proteasome inhibitors. FOXO1 is ubiquitinated in vivo and in vitro; insulin enhances its ubiquitination in cells. In addition, the insulin signal to FOXO1 degradation is mediated by the PI3K pathway. FOXO1 mutates at the serine or threonine residues that are phosphorylated by PKB inhibit FOXO1 ubiquitination in vivo and in vitro. These data suggest that efficient ubiquitination of FOXO1 requires both phosphorylation and cytoplasmic retention in the cells (Matsuzaki et al., 2003).

Skp2 is an oncogenic subunit of the Skp1/Cul1/F-box protein ubiquitin complex. It inhibits FOXO1 during tumor suppression through ubiquitin-mediated degradation but it only ubiquitinates and degrades FOXO1, not FOXO3A or FOXO4. The effect of Skp2 on FOXO1 requires PKB-specific phosphorylation of FOXO1 at Ser-256. Decrease ubiquitination is seen in the mutant of FOXO1 in which all three PKB sites are replaced by alanine and FOXO1 is forced to remain in the cytoplasm, through a mutation in the
NLS. In addition, when phosphorylated FOXO1 is confined to the nucleus by a mutation in the NES, FOXO1 ubiquitination is decreased (Huang et al., 2005). This study also confirmed that only cytoplasmic FOXO1 is successfully ubiquitinated by an E3 ubiquitin ligase and subsequently degraded.

IKKβ (I kappa B kinase β) also causes the proteasome-dependent degradation of FOXO factors. IKKβ induces the phosphorylation of FOXO3A at Ser644, in the extreme C-terminal portion of the molecule. This phosphorylation results in the ubiquitination and subsequent degradation of FOXO3A. Since IKKβ-induced tumorigenesis can be suppressed by overexpression of FOXO3A (Hu et al., 2004), the regulation of FOXO protein degradation by IKKβ may play an important role in tumorigenesis. However, Ser644 is not conserved in other FOXO isoforms and is not present in worms and flies. It remains to be determined whether IKKβ phosphorylates and controls the other FOXO isoforms. It is possible that the degradation of FOXO isoforms is regulated by different protein kinases through other independent mechanisms.

Recently, it was showed that in the presence of oxidative stress FOXO4 becomes monoubiquitinated, resulting in its re-localization to the nucleus and increased transcriptional activity (van der Horst et al., 2006). This study demonstrated that besides FOXO degradation, the ubiquitination of FOXO may also have other biological functions.

1.4.3.2. Deubiquitination of FOXO

USP7/HAUSP is a herpes virus-associated ubiquitin-specific protease. Deubiquitination of FOXO4 requires the enzyme USP7/HAUSP, which interacts with and deubiquitinates FOXO4 in response to oxidative stress. Oxidative stress-induced ubiquitination and deubiquitination by USP7 do not influence the half-life of FOXO4.
protein. Moreover, USP7 does negatively affect FOXO transcriptional activity towards endogenous promoters (van der Horst et al., 2006).

2. Ubiquitin, Proteasome and MDM2 as an E3 Ligase

Ubiquitination is a reversible post-translational modification of cellular proteins, in which ubiquitin is attached to the target proteins. Aberrations in the ubiquitination system are implicated in the pathogenesis of many diseases, including certain malignancies, neurodegenerative disorders and pathologies of the inflammatory immune response. Deubiquitination is a biological process in which one or more ubiquitin moieties are removed from protein. Deubiquitination helps to maintain the pool of free ubiquitin.

2.1. Ubiquitin-Proteasome System

2.1.1. Ubiquitin and Ubiquitination

Ubiquitin (Ub) is a small protein composed of 76 amino acids. Ub is found only in eukaryotic organisms and not in either eubacteria or archaeabacteria. Among eukaryotes, ubiquitin is highly conserved. Ub is a heat-stable protein folded into a compact globular structure. Ub is found throughout the cell and can exist either in free form or as part of a complex with other proteins. In the latter case, Ub is conjugated to proteins through a covalent bond between glycine at the C-terminal end of Ub and the side chains of lysine on the target proteins. A single Ub can be conjugated to the lysine of these proteins, or more commonly, Ub-chains can be attached. Ubiquitination depends on ATP.

Ub is encoded by a family of genes whose translation products are fusion proteins. The Ub genes typically exist in two states: 1) The ubiquitin gene can be fused to a ribosomal gene giving rise to a translation product that is an Ub-ribosomal fusion protein; or 2) Ub genes can exist as a linear repeat, meaning that the translation product consists of a linear chain of Ub-molecules fused together (a polyubiquitin molecule).
After the fusion proteins are synthesized, another protein called Ub-C-term hydrolase cleaves the fusion proteins at the C-terminal end of Ub. This either liberates an individual Ub and ribosomal protein or liberates a set of Ub monomers from the polyubiquitin.

Ubiquitination is a process in which ubiquitin is conjugated to the protein substrates. The attachment of a single ubiquitin polypeptide, monoubiquitin, to a substrate serves as an important regulatory modification (Hicke, 2001). Monoubiquitin acts as a sorting signal throughout the endocytic pathway and regulates diverse proteins including histones, endocytic machinery and transcription factors. Monoubiquitination targets proteins to the lysosome, either by directing endocytosis of cell-surface receptors or by sorting newly synthesized hydrolases from the Golgi to their resident lysosomal compartment (Lee and Tyers, 2001). A polyubiquitin chain is formed when ubiquitin is attached to a lysine within ubiquitin itself and this process is repeated. Polyubiquitin chains linked through different lysine residues are involved in distinct cellular functions. For instance, the signal necessary for degradation of substrates by the proteasome is a polyubiquitin chain attached through Lys-48, whereas chains linked through Lys-63 are crucial to the role of ubiquitin in DNA damage repair (Pickart, 2001). Polyubiquitination typically targets proteins for rapid proteasomal degradation. Polyubiquitination is also critical in protein quality control, where it helps to eliminate improperly processed or misfolded proteins from the ER (endoplasmic reticulum), in a process called ER-associated degradation (Lee and Tyers, 2001).

2.1.2. **Deubiquitination**

Both poly- and monoubiquitination can be reversed by DUBs (deubiquitinating enzymes) that specifically cleave the isopeptide bond at the C terminus of ubiquitin. DUBs also generate the pool of free ubiquitin both by liberating ubiquitin from precursor
ubiquitin fusion proteins and by recycling ubiquitin from the branched polyubiquitin chains of degraded proteins (D'Andrea and Pellman, 1998; Wilkinson, 1997).

The DUBs are comprised of two groups of enzymes, the UCHs (ubiquitin C-terminal hydrolyases) and the USPs (ubiquitin-specific proteases; referred to as UBPs in yeast). The UCHs are small (20-30 kDa), closely related proteases that are generally involved in cleaving ubiquitin from small processed peptides. The USPs are more numerous, much larger (60–300 kDa), and are thought to have specific protein targets. USPs can be identified by conserved sequences within the active site, but sequences outside of the catalytic domain are highly divergent, likely reflecting their role in mediating interactions with different protein targets (Hu et al., 2002; Wilkinson, 1997; Wilkinson et al., 1995).

There are 16 known UBPs in *Saccharomyces cerevisiae*, and 63 putative USP genes in humans. Single and even multiple UBP deletions in yeast generally produce minimal phenotypic abnormalities, suggestive of functional redundancies among the yeast UBP family (Amerik et al., 2000). However, studies have shown that USPs can play specific roles in various biological processes in higher eukaryotes, suggesting a more specialized role as cellular regulators in multicellular organisms. Specific DUBs have been shown to regulate eye development (Huang et al., 1995; Huang and Fischer-Vize, 1996), cell growth in response to cytokines (Zhu et al., 1996), oncogenic transformation (Gray et al., 1995; Jensen et al., 1998; Papa and Hochstrasser, 1993), cell cycle regulation (Hu et al., 2002), chromatin structure (Robzyk et al., 2000), and transcriptional regulation (Holowaty et al., 2003; Mimnaugh et al., 1997; Moazed and Johnson, 1996).
2.1.3. Ubiquitination Machinery and Proteasome Pathway

Ubiquitination of a protein substrate requires the concerted action of three classes of enzymes designated E1, E2, and E3. E1 (ubiquitin activating enzyme) initially activates ubiquitin in an ATP-dependent reaction through the formation of a thiol-ester bond between the carboxyl terminus of ubiquitin and the thiol group of a specific cysteine residue of E1. Ubiquitin is then transferred to a specific cysteine residue on one of several E2 (ubiquitin-conjugating enzymes, Ubcs). E2 enzymes in turn transfer the ubiquitin either directly to a substrate or to the final class of enzymes known as E3 (ubiquitin protein ligases). E3 enzymes catalyze the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the amino group of lysine residues on the target protein. A substrate may then undergo multiply ubiquitinations through the attachment of additional ubiquitin molecules to specific lysine residues of ubiquitin itself. In many cases the E1, E2, and E3 enzymes form large, multi-protein complexes. This increases the efficiency of the process by allowing the rapid thiol-ester transfer of ubiquitin molecules between proteins.

This type of protein degradation plays a role in many cellular processes, such as cell cycle regulation, antigen presentation, and the disposal of denatured, unfolded, or oxidized proteins. Most intracellular protein degradation is through the ubiquitin-proteasome pathway (Ciechanover and Schwartz, 2002; Hershko and Ciechanover, 1998).

In the ubiquitin-proteasome degradation pathway, the covalent attachment of multiple ubiquitin molecules to lysine residues of a target protein serves to signal its recognition and rapid degradation by the 26S proteasome. The proteasome is a large, multisubunit complex that exists in cells in two forms: a 20S and a 26S species. The
Figure 3  Ubiquitin-proteasome degradation pathway. E1 (ubiquitin activating enzyme) activates Ub in the presence of ATP. Activated Ub is then transferred to E2 (ubiquitin conjugating enzyme). E2 in turn transfers Ub to E3 (ubiquitin protein ligase). E3 binds ubiquitin to the substrate protein. Ubiquitinated proteins are degraded by the proteasome. *Ub* – ubiquitin.
active protease sites sequestered in its central cavity, so that only proteins entering this chamber are degraded. The openings to this cavity permit only denatured proteins to enter, where they are progressively cleaved to small peptides. The addition of a 19S regulator to either or both ends of the 20S proteasome creates the 26S proteasome. The 19S regulator contains ATPases and other proteins and serves numerous functions, including the recognition of the substrate and its translocation to the catalytic core (Voges et al., 1999).

2.2. MDM2 as an Ubiquitin E3 Ligase

2.2.1. General Information about MDM2

MDM2 is the product of the 'murine double minute 2' gene. The MDM2 gene was originally identified as one of three genes (mdm1, 2, 3) which were overexpressed by more than 50-fold through amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM). The mdm2 genes are located on small, acentromeric extrachromosomal nuclear bodies, called double minutes, which are retained in cells only if they provide a growth advantage. The gene product of mdm2 was later shown to be responsible for cell transformation when MDM2 was overexpressed (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). In 1992, Oliner cloned the human MDM2 gene and mapped it to chromosome 12q13-14 (Oliner et al., 1992). Both the mdm2 gene and its human counterpart, MDM2, consist of 12 exons that can generate many different MDM2 proteins, as shown in Figure 4. There are two different promoters, the second of which is responsive to p53. These promoters generate two proteins, the full-length p90 and a shorter p76 protein that initiates at an internal ATG (Olson et al., 1993; Perry et al., 1993; Saucedo et al., 1999). p76 is missing part of the p53-binding domain and it can act as a dominant negative inhibitor of p90 and activate p53. Alternative splicing of
MDM2 to generate shorter proteins also occurs in many human and mouse tumors. More than 40 different alternatively spliced transcript variants have been isolated from both tumor and normal tissues. In humans, MDM2-a and MDM2-b are the major splice variants that delete exons 4–9 and 4–11, respectively. Neither product contains the p53-binding motif. MDM2-b, also named MDM2-ALT1, interacts with full-length MDM2 and sequesters it in the cytoplasm (Bartel et al., 2002).

2.2.2. Functions of MDM2

2.2.2.1. MDM2 and Cell Cycle

Overexpression of MDM2 has been shown to correlate with the cyclin-dependent kinase inhibitor p21. In breast cancer cells, overexpression of MDM2 correlates with lack of p21 expression (Jiang et al., 1997). On the other hand, in squamous cell carcinoma, overexpression of MDM2 is associated with high levels of p21 (Ng et al., 1999).

MDM2 reverses the growth inhibition at G1 imposed by p53 and RB (Chen et al., 1996; Xiao et al., 1995). Overproduction of MDM2 can overcome the TGF-β-imposed growth inhibition via the RB–E2F pathway (Sun et al., 1998).

Transgenic mice experiments showed that expression of a BLG (β-lactoglobulin)/mdm2 transgene (BLGmdm2) in the epithelial cells of the mouse mammary gland caused mammary epithelial cells to undergo multiple rounds of S phase without cell division, and resulted in polyploidy and tumor formation. The effect of MDM2 on S phase is independent of the p53 status (Lundgren et al., 1997).

2.2.2.2. MDM2 and Differentiation

One phenotype of tumor cells is the lack of terminal differentiation. MDM2 plays a very important role in epidermal differentiation (Dazard et al., 1997). MDM2 overexpression in the granular layer perturbs the differentiation program (Alkhalaf et al.,...
In rhabdomyosarcoma, forced expression of MDM2 inhibits MyoD function and consequently inhibits muscle differentiation (Fiddler et al., 1996). A MDM2-conditional mice experiment showed that MDM2 plays a very important role in bone organogenesis and homeostasis through inhibition of p53 function, which is a prerequisite for master osteoblast transcriptional regulator Runx2 activation, osteoblast differentiation, and proper skeletal formation (Lengner et al., 2006). NUMb is a cell fate determinant protein. MDM2 associates with NUMb and influences cell differentiation and survival through translocation of NUMb to nucleus and degradation of NUMb (Juven-Gershon et al., 1998).

However, microinjection of MDM2 mRNA in two-cell stage zebrafish embryos caused inhibition of cellular convergence during gastrulation. Clones derived from MDM2 microinjected blastomeres were significantly smaller than those derived from control microinjections. This indicates that MDM2 expression may be important during the differentiation of neural and muscular tissues of zebrafish (Thisse et al., 2000).

2.2.2.3. MDM2 and Ribosome Biogenesis

Ribosome biogenesis is the process of making ribosomes. It takes place both in the cell cytoplasm and in the nucleolus of eukaryotic cells. It involves the coordinated action of over 200 proteins in the synthesis and processing of the four rRNAs, as well as assembly of those rRNAs with the ribosomal proteins. Proper ribosome assembly is essential for the health of a cell.

Ribosome proteins L5, L11 and L23 exist in the same complex with MDM2 in response to ribosome stress, such as exposure to actinomycin D. They activate p53 by inhibiting MDM2-mediated p53 suppression (Dai et al., 2004; Lohrum et al., 2003; Marechal et al., 1994; Zhang et al., 2003).
2.2.2.4. MDM2 and Transcription

MDM2 affects the gene transcription by inhibiting p53-mediated transactivation (Momand et al., 1992). MDM2 uses multiple mechanisms to inactivate p53 and to inhibit its transcriptional activity. MDM2 targets p53 for ubiquitination and degradation by the proteosome, shuttles p53 out of the nucleus, prevents its interaction with transcriptional coactivators and recruits the known transcriptional corepressors, such as hCtBP2, to p53.

2.2.2.5. MDM2 and Protein Ubiquitination and Degradation

E3 ubiquitin ligases are a large family of proteins engaged in the regulation of the turnover and activity of many proteins. Together with ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2, E3 ubiquitin ligases catalyze the ubiquitination of a variety of biologically significant protein substrates leading to their degradation through the 26S proteasome. Because they serve as the specific substrate-recognition element of the system, E3 ligases play an important role in the ubiquitin-mediated proteolytic cascade. There are approximately 1000 E3 ligases in the human genome.

MDM2 possesses the activity of an E3 ubiquitin ligase. MDM2 was initially found to promote the proteasome–dependent degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). It functions as an E3 ubiquitin ligase for p53 and for itself through its RING finger domain at the C-terminus (Fang et al., 2000; Honda et al., 1997; Honda and Yasuda, 2000). It is now known that MDM2 also promotes the degradation of several other proteins in intact cells, such as: Numb (Yogosawa et al., 2003), RB (Miwa et al., 2006; Uchida et al., 2005) and MDMX (Pan and Chen, 2003).
2.2.3. Regulation of MDM2 E3 Activity

2.2.3.1. Sumoylation of MDM2

2.2.3.1.1. SUMO and Sumoylation

SUMOs (small ubiquitin-related modifiers) constitute a family of highly conserved proteins found in all eukaryotes and are required for viability of most eukaryotic cells, including budding yeast, nematodes, fruit flies, and vertebrate cells in culture (Apionishev et al., 2001; Epps and Tanda, 1998; Fraser et al., 2000; Hayashi et al., 2002; Jones et al., 2002). In multicellular organisms, SUMO conjugation takes place in all tissues and at all developmental stages (Chen et al., 1998; Howe et al., 1998; Joanisse et al., 1998; Kamitani et al., 1998; Kurepa et al., 2003; Lois et al., 2003; Mannen et al., 1996; Shen et al., 1996). Since its discovery in 1996, SUMO has been found covalently attached to more than 50 proteins, including the androgen receptor, IκBα, c-Jun, HDACs and p53. Proteins that participate in transcription, DNA repair, nuclear transport, signal transduction and the cell cycle have been found to be sumoylated. In contrast to ubiquitination, however, sumoylation of a protein does not appear to target it for rapid degradation, but rather affect the ability of the modified protein to interact with cellular factors. Most SUMO-modified proteins that have been characterized in mammalian systems are involved in transcription and they are often repressed by SUMO conjugation. However, genetic studies in model organisms have pointed to a role for SUMO in chromosome dynamics and higher order chromatin structures, illustrating the diversity of SUMO function.

SUMO often has a positive effect on protein-protein interactions, and it promotes assembly of several multi-protein complexes. However, the effects of SUMO on interactions vary depending on the substrates. SUMO can also act by a completely
different mechanism, including the prevention of ubiquitination of a protein by blocking lysine residue where Ub would normally be attached (Desterro et al., 1998; Hoege et al., 2002; Lee et al., 2003; Lin et al., 2003a).

The linkage between SUMO and its substrates is an isopeptide bond between the C-terminal carboxyl group of SUMO and the ε-amino group of a lysine residue in the substrate. A three-step enzyme pathway attaches SUMO to specific substrates, and other enzymes cleave SUMO off its targets. The enzymes of the SUMO pathway, although analogous to those of the Ub pathway, are specific for SUMO and have no role in conjugating Ub or any of the other ubiquitin-like modifiers. The SUMO pathway begins with a SUMO-activating enzyme (also called E1). E1 catalyses an ATP-dependent activation of the SUMO C-terminus and then transfers activated SUMO to a SUMO-conjugating enzyme (E2), also known as Ubc9. SUMO is then transferred from Ubc9 to the substrate with the assistance of one of several SUMO-protein ligases (E3s). In contrast to the ubiquitin-conjugating system, where E3 ligase is responsible for target recognition, the recognition of SUMO targets is mediated by both E2 and E3 enzymes. Many of the Lys residues where SUMO becomes attached are in the short consensus sequence \( \psi \Omega KXE/D \), where \( \psi \) denotes a bulky aliphatic residue, \( \Omega \) denotes a large hydrophobic amino acid, generally isoleucine, leucine, or valine; K is the lysine residue being modified; X is any residue; and E/D is a glutamic or aspartic acid. This motif is bound directly by Ubc9. E3 ligases probably enhance specificity by interacting with other features of the substrate. Although most known SUMO targets contain this sequence, other conjugation sites are now beginning to be known, such as TKET in \( S. \text{cerevisiae} \) PCNA (Hoege et al., 2002) and VKYC in Smad4 (Lee et al., 2003; Lin et al., 2003b).

Sumoylation is a reversible modification, and removal of SUMO is carried out by enzymes that specifically cleave at the C-terminus of SUMO (Johnson, 2004). All known
SUMO-cleaving enzymes belong to the family of ubiquitin like proteases and contain a 200 amino acid C-terminal core domain (the Ulp domain). The core domain has the SUMO cleaving activity and contains the catalytic triad Cys-His-Asn (Mossessova and Lima, 2000). Mammals have seven members of the Ulp1 family: SENP1-3 and SENP5-8. Only four of the SENP genes have been confirmed to encode SUMO proteases, namely SENP1 (Bailey and O'Hare, 2002), SENP3 (SMT3IP1) (Nishida et al., 2000), SENP6 (SUSP1) (Kim et al., 2000) and SENP2 (Axam, SMT3IP2/Axam2, SuPr-1) (Best et al., 2002; Kadoya et al., 2002; Nishida et al., 2001).

2.2.3.1.2. Sumoylation and MDM2

SUMO-1 modification of MDM2 can differentially modulate the outcome of MDM2 E3 ligase activity in a manner that favors accumulation of p53. Upon Sumo-1 conjugation, MDM2 is protected from self-ubiquitination and elicits greater ubiquitin-protein isopeptide ligase (E3) activity toward p53, thereby increasing its oncogenic potential. This switch in modification status is stress-responsive, because UV irradiation leads to a decrease in the interaction of MDM2 with Ubc9 and a corresponding loss of MDM2 sumoylation (Buschmann et al., 2001). Further studies showed that Ubc9 can associate with MDM2 only if amino acids 40-59 within the N-terminus of MDM2 are present. Furthermore, addition of a peptide corresponding to amino acids 40-59 of MDM2 efficiently inhibits MDM2 sumoylation in vitro and in vivo (Buschmann et al., 2001)

2.2.3.2. Ubiquitination and Degradation of MDM2

As mentioned earlier, MDM2 mediates autoubiquitination as well as the ubiquitination of other substrates. The balance between auto- and substrate-ubiquitination of MDM2 is modulated physiologically by posttranslational modifications, including sumoylation and phosphorylation. After SUMO conjugation to MDM2, MDM2
E3 ligase activity is shifted toward p53, while self-ubiquitination is minimized (Buschmann et al., 2001).

P300 is an acetylase-possessing transcriptional co-activator that has been shown to mediate transcription by numerous transcriptional activators. It binds to and stabilizes MDM2. It stabilizes MDM2 by retaining it in a specific nuclear structure but does not acetylate MDM2 in solution or in cells.

2.2.3.3. Phosphorylation of MDM2

The first demonstration of the complex nature of MDM2 phosphorylations was by Henning who showed that the phosphorylation status of MDM2 was influenced by early gene expression of SV40. MDM2 is stabilized in the presence of SV40. Moreover, hyperphosphorylated MDM2 participates in a trimeric complex with p53 and T-Ag (T-antigen, the transforming protein of SV40), which is thought to activate oncogenic functions of MDM2 and enhance the transforming potential of T-Ag (Henning et al., 1997).

Nearly 20% of the residues of MDM2 are either serine or threonine. MDM2 protein is phosphorylated at multiple sites in vivo. Two clusters of phosphorylation sites are located at the N-terminal (amino acids 1–193) and central amino acids 194–293 of murine Mdm2, respectively (Hay and Meek, 2000).

2.2.3.3.1. DNA-PK, ATM and MDM2

The PI3K family of enzymes generates lipid 'second messengers' that mediate signal transduction. It includes four classes of proteins. Class IV of PI3K includes mTOR (mammalian target of rapamycin), DNA-PK (DNA activated protein kinase), ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) protein kinases. MDM2 is phosphorylated in vitro by both DNA-PK (Mayo et al., 1997) and ATM (Maya et al., 2001)
but phosphorylation by ATR has not yet been reported. Of eight potential DNA-PK targets in MDM2, only Ser17 has been shown to be phosphorylated by this enzyme in vitro (Mayo et al., 1997). Although physiological phosphorylation of Ser17 has been confirmed, the phosphorylation site itself has been reported to have a significant impact on the ability of MDM2 to regulate the response to p53. S17A mutant, mimics the dephosphorylation form of MDM2, was significantly more effective in inhibiting p53-dependent transactivation in cultured cells than wild-type MDM2. Nuclear magnetic resonance studies also showed that MDM2 amino acids 16–24 can form a "flexible lid" that folds over and stabilizes the MDM2 structure but competes only weakly with p53 for binding to this cleft. The S17D mutant peptide which mimics the constitutive phosphorylation form was found to have higher affinity for MDM2 than the wild-type peptide (McCoy et al., 2003).

ATM is able to phosphorylate MDM2 at Ser395 in vitro. In response to ionizing radiation and radiomimetic drugs, MDM2 undergoes rapid ATM-dependent phosphorylation prior to p53 accumulation, which results in a decrease in its reactivity with the 2A10 monoclonal antibody. MDM2 S395D is impaired in promoting p53 degradation and it is markedly less able to promote p53 cytoplasmic export (Maya et al., 2001).

2.2.3.3.2. PKB and MDM2

Mitogen-induced activation of PKB results in phosphorylation of MDM2 on Ser166 and Ser186. Phosphorylation on these sites is necessary for translocation of MDM2 from the cytoplasm into the nucleus (Mayo and Donner, 2001). Moreover, phosphorylation of MDM2 not only enhances its nuclear localization but its interaction with p300, and inhibits its interaction with p19ARF, resulting in increased p53 degradation (Zhou et al., 2001).
PKB inhibits MDM2 self-ubiquitination via phosphorylation of MDM2 on Ser166 and Ser188. Stimulation of human embryonic kidney 293 cells with IGF-1 increased MDM2 phosphorylation on Ser166 and Ser188 in a PI3K-dependent manner. Treatment of both human embryonic kidney 293 and COS-1 cells with PI3K inhibitor LY-294002 led to proteasome-mediated MDM2 degradation (Feng et al., 2004).

2.2.3.3. c-Abl and MDM2

c-Abl is an non-receptor tyrosine kinase that can shuttle between the cytoplasmic and nuclear compartments. In response to stress, such as DNA damage, c-Abl promotes cell growth arrest and apoptosis. The apoptotic activity of c-Abl is mediated partly via p73 (Agami et al., 1999; Gong et al., 1999) and to a lesser extent through a p53-dependent pathway (Yuan et al., 1997). One mechanism for the protection of p53 by c-Abl is that c-Abl can neutralize the ability of MDM2 to ubiquitinate p53 and degrade it (Sionov et al., 1999). C-Abl directly interacts with MDM2 at multiple sites in the nucleus, enhances its accumulation in a p53-independent manner. c-Abl phosphorylates MDM2 at Tyr394. Substitution of Tyr394 by Phe394 enhances the ability of MDM2 to promote p53 degradation and to inhibit the transcriptional and apoptotic activities of p53 (Goldberg et al., 2002).

2.2.3.4. CK2 and MDM2

Protein kinase CK2 is a ubiquitous Ser/Thr protein kinase required for cell cycle progression and cell viability. Serine residue at position 269 of MDM2 was established as the most important CK2 phosphorylation site by analyses with deletion mutants of MDM2 and a peptide library. Phosphorylation of MDM2 by CK2 is stimulated in the presence of the C-terminal sequences of p53, but binding studies revealed that the
Figure 4  Structure of MDM2 gene and protein
Figure 4  Structure and regulation of MDM2 gene and protein. MDM2 gene consists of 12 exons. Two promoters are shown by arrows. Full-length MDM2 p90 is translated from the first start codon ATG in exon 3 and the short form, p76, is translated from the second ATG in exon 4. Phosphorylation sites are indicated by the letter P within an ellipse. Their locations relative to the functional domains of MDM2 are shown. Protein kinases, where known, are indicated in boxes with the target residue(s) shown above. Five major alternative splice variants MDM2-a, MDM2-b, MDM2-c, MDM2-d, and MDM2-e are shown. Full-length Mdm2 and known motifs are also represented. **NLS**-nuclear localization signal; **NES**-nuclear export signal; **Zn-finger**-zinc finger domain; **NoLS**-nucleolar localization signal; **RING-finger**-ring finger domain. The numbers above the drawings denote amino acid numbers and roman numerals denote the exon numbers.
biological function of CK2 phosphorylation still needs to be established by further studies because phosphorylation of MDM2 at Ser269 does not have any influence on the binding of p53 to MDM2 (Gotz et al., 1999).

3. Functional interaction among FOXO, p53, and MDM2

3.1. Interaction between MDM2 and p53

P53 is a transcription factor that regulates the cell cycle and functions as a tumor suppressor. p53 has been described as "the guardian of the genome" or the "master watchman", referring to its role in conserving stability by preventing genome mutations. In the absence of genetic damage, p53 is a very unstable protein with a half-life ranging from 5-30 min and transcriptional activity is inert (Yuan et al., 1996). In the presence of stress, such as DNA damage, hypoxia, telomere shortening, and oncogenic activation, p53 becomes stable and activated by blocking its degradation (Caspari, 2000; Meek, 1994; Sakaguchi et al., 1998; Siliciano et al., 1997). p53 can kill cells via dual transcription-dependent and -independent functions in the nucleus and mitochondria (Mihara et al., 2003; Vousden and Lu, 2002).

The p53-MDM2 system forms a feedback loop, in which p53 upregulates MDM2 by activating MDM2 transcription (Barak et al., 1993). Experiments with knock-out mice revealed that deletion of the mdm2 gene results in embryonic lethality, which can be rescued by deletion of the p53 gene (Jones et al., 1995; Montes de Oca Luna et al., 1995). Inhibition of cell growth and marked cell death is often seen in the absence of p53 regulation by MDM2, further emphasizing the importance of the p53–MDM2 autoregulatory loop in controlling of cell growth and death.

MDM2 negatively regulates p53 in several ways:
1) MDM2 binds to p53 and this interaction is conformation based. Site-directed experiments have demonstrated the importance of p53 residues Leu14, Phe19, Leu22, and Trp23 (Lin et al., 1994). A minimal MDM2-binding site on p53 residues 18-23 was mapped with p53-derived peptides (Picksley et al., 1994). On binding to the p53 transactivation domain, MDM2 inhibits its transcriptional activity. Crystallographic data showed that the amino terminal domain of MDM2 forms a deep hydrophobic cleft into which the transactivation domain of p53 binds, thereby concealing itself from interacting with the transcriptional machinery (Kussie et al., 1996).

2) MDM2 functions as an E3 ubiquitin ligase for p53 (Honda and Yasuda, 1999; Lohrum et al., 2000; Tao and Levine, 1999; Weber et al., 1999). The level of MDM2 is very important for the ubiquitination level of p53. In vitro studies showed that low levels of MDM2 activity induce monoubiquitination, whereas high levels promote polyubiquitination and nuclear degradation of p53 (Li et al., 2003a).

3) MDM2 promotes the export of p53 from the nucleus. p53 shuttles between nucleus and cytoplasm in the cells in response to stress. MDM2 contains an NES, but the study showed that the MDM2 RING finger domain, not the MDM2 NES, is necessary for the efficient export of p53 to the cytoplasm (Boyd et al., 2000; Geyer et al., 2000). Another study also showed that low MDM2 levels induce cytoplasmic translocation of p53 (Li et al., 2003a), whereas MDM2-mediated p53 monoubiquitylation promotes its mitochondrial translocation (Marchenko et al., 2007).

3.2. Interaction between p53 and FOXO

p53 and FOXO factors share similar characteristics. Both are involved in stress response and can be post-translationally modified by phosphorylation and acetylation. They also have some common downstream targets, such as: Fas ligand (Greer and
Brunet, 2005), GADD45 (Greer and Brunet, 2005), PA26 (Greer and Brunet, 2005), p21 (Seoane et al., 2004) and PUMA (You et al., 2006a) (Figure 5).

Recently it was shown that p53 and FOXO3A interact with each other. In response to oxidative stress, p53 binds to FOXO3A. In vivo, these two transcription factors exhibit “crosstalk”. In response to DNA damage, p53 activation leads to FOXO3A phosphorylation and subcellular localization change, which results in inhibition of FOXO3A transcription activity. PKB is dispensable for p53-dependent suppression of FOXO3A. By contrast, SGK1 was significantly induced in a p53-dependent manner after DNA damage, and this induction is through extracellular signal-regulated kinase 1/2-mediated posttranslational regulation. Furthermore, inhibition of SGK1 expression by a small interfering RNA knockdown significantly decreased FOXO3A phosphorylation in response to DNA damage.

Nuclear activated FOXO3A can impair p53 transcriptional activity. However, activation of FOXO3A either by serum starvation or by expressing a constitutively active form of FOXO3A can induce p53-dependent apoptosis, even in cells bearing a transcriptionally inactive form of p53 (You et al., 2006b).
Figure 5  Stress-induced FOXO and p53 pathway.
HYPOTHESIS & OBJECTIVES

FOXO factors are known to be ubiquitinated, but so far no general E3 ubiquitin ligases capable of ubiquitination of all FOXO factors have been identified. Skp2 is reported to promote the ubiquitination and degradation of FOXO1, but this effect is limited to FOXO1. A genetics study in *C. elegans* showed that skp expression is actually needed for FOXO transcriptional activity.

Since MDM2 is known to be an E3 ubiquitin ligase for p53 and both p53 and FOXO factors are important regulators in stress responses, aging and tumorigensis, we, therefore, hypothesize that MDM2 interacts with and promotes the ubiquitination and degradation of FOXO factors.

To substantiate the hypothesis, my thesis studies are to achieve the following objectives:

1. Determine whether FOXO factors and MDM2 interact;
2. Determine whether MDM2 regulates the transcriptional activity of FOXO factors;
3. Determine whether MDM2 decreases the stability of FOXO factors;
4. Determine whether MDM2 changes the biological function of FOXO;
5. Identify the signals that regulate effect of MDM2 on FOXO and investigate the functional relationship among p53, FOXO and MDM2.
MATERIALS AND METHODS

Chemicals, Antibodies and Cell Lines:

Cycloheximide and nicotinamide were purchased from Sigma and MG132 from Calbiochem. Antibodies against FOXO1 (H-128, Santa Cruz Biotechnology), FOXO3A (H-144, Santa Cruz Biotechnology), MDM2 (SMP14, Santa Cruz Biotechnology; 2A10, Calbiochem), c-Myc (A-14, Santa Cruz Biotechnology), Flag (M2-A-2220 and F-7452, Sigma), HA (MMS-101P and PRB-101P, Covance), MnSOD (Upstate), TRAIL (BD Pharmingen), p27 (N-20, Santa Cruz), acetyl-K (Upstate), α-Tubulin (Sigma), and β-actin (AC-74, Sigma) were purchased from commercial sources.

H1299/V138 cells were cultured as described (Pochampally et al., 1999). HEK 293T, NIH 3T3, DU145, JCA1, PC3, HeLa, MCF-7, Saos-2, H1299, p53 null MEFs (mouse embryonic fibroblasts) and p53 and MDM2 double null MEFs (Huang et al., 2005; Peng et al., 2001) were maintained in DMEM (Dulbecco’s modified Eagle’s medium) with 10% FBS (fetal bovine serum). LNCaP cells were cultured in RPMI 1640 with 10% FBS at 37°C. H1299 cells stably expressing human MDM2 were generated by cotransfecting MDM2 with pcDNA3 and selecting in the presence of 750 μg/ml G418.

Plasmids:

pcDNA3-Flag-FOXO1, pcDNA3-Flag-FOXO1 (AAA), HA-FOXO3A, HA-FOXO3A (AAA) (Li et al., 2003b), HA-SIRT1 (Yang et al., 2005), HA-SIRT1(734R) (Yang et al., 2007), pcMDM2 and different MDM2 mutants were described previously (Armoni et al.,
2006; Chen et al., 1995; Chen et al., 1993; Freedman and Levine, 1999). To construct pcDNA3-HA-FOXO1 (1-150), FOXO1 cDNA fragment coding the first 150 amino acids were amplified by PCR with primers 5'-CGG GGG TCA CCG GAT CCA TGG CCG AGG C-3' and 5' –GCG GCG GGA CGA TCT AGA CTA GCG CGG CTG C-3', which generated BamHI and Xbal sites at 5' and 3' ends of the DNA fragment, respectively. The amplified FOXO1 (1-150) fragment was cloned into the BamHI and Xbal sites of pcDNA3.1 HA vector (Invitrogen). pcDNA3-HA-FOXO1 (1-270) and pcDNA3-HA-FOXO1 (256-655) were constructed similarly by generating a BamHI site at the 5' end and a Xbal site at the 3' end of the corresponding FOXO1 cDNA fragments. The upstream primer of FOXO1 (1-270) was same as the FOXO1 (1-150), and the downstream primer was 5'-CTT GGC TCT AGA AGC TCG GCT TCG GCT CTT AG -3'. The upstream primer of FOXO1 (256-655) was 5'-GGA GAA GAG CTG GAT CCA TGG ACA ACA AC-3' and the downstream primers was 5'-CGG GCC CTC TAG ATC AGC CTG ACA CC -3'. MDM2 siRNAs were subcloned into pSilencer-Neo (Ambion). The corresponding oligonucleotides for generating the MDM2 siRNA were 5'-GAT CCG CAG GTG TCA CCT TGA AGG TTT CAA GAG AAC CTT CAA GGT GAC ACC TGT TTT TTG GAA A-3'and 5'-GAT CCG TGG TTG CAT TGT CCA TGG CTT CAA GAG AGC CAT GGA CAA TGC AAC CAT TTT TTG GAA A-3'. The oligonucleotides for GFP siRNA were from Ambion.

**Transfections and Immunological Assays:**

For co-precipitation analysis, 10⁶ cells were plated in 100 mm dishes in a medium containing 10% fetal bovine serum. One day after plating, cells were transfected with the indicated plasmids by Lipofectamine Plus following the protocol from Invitrogen. Cellular extracts were prepared in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10
mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. After pre-clearance by incubating with protein A-agarose for 1 hour (h) followed by brief centrifugation, the extracts were incubated sequentially with 1-3 µg antibody and protein G-agarose beads for 4 h at 4°C. After four times washes with the lysis buffer, the immunoprecipitates were eluted from the beads by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer.

For immunoblotting, cellular extracts or immunoprecipitates were separated on SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, probed with the cognate antibody and visualized with enhanced chemiluminescence.

For immunofluorescence analyses, H1299 cells or MEFs transfected cells on cover slips were cultured in DMEM for 16 h, washed once with PBS (phosphate buffered saline) and fixed in 4% paraformaldehyde for 10 min at 37°C. The cells were permeabilized in buffer containing 1% Triton X-100 and 1% BSA at room temperature for 30 min and incubated for another 1 h in PBS containing 0.2% NP-40, 1% bovine serum albumin and the primary antibody. After washing three times in PBS, the cells were incubated for 45 min with goat anti-mouse IgG conjugated with Alexa Fluor 594 (red) or fluorescein isothiocyanate (FITC; green) conjugated anti-rabbit IgG (Molecular Probes), respectively and washed three times with PBS. The slides were dried and mounted with Vectashield mounting medium with DAPI (4’, 6’-diamidino-2-phenylindole). DAPI staining was performed to visualize the nucleus. Regular fluorescent microscopic images were obtained with a Nikon Diaphot microscope using a Photometrix PXL cooled CCD camera. The microscope was equipped with the appropriate filters for three-color imaging and a motorized stage for obtaining z-series images. Digital image files were processed and deconvolved using the Oncor Image software (Oncor Inc.). High-resolution images of the deconvolved and 3-D reconstructed image z-series stacks were
processed for presentation with Adobe Photoshop. For confocal analysis, samples were viewed with a Leica DMI6000 inverted microscope, TCS SP5 confocal scanner, and a 100X/1.40NA Plan Apochromat oil immersion objective (Leica Microsystems, Germany). 405 Diode and HeNe 594 laser lines were applied to excite the samples and tunable filters were used to minimize spectral overlap between fluorochromes. DIC imaging was performed using an argon laser line. Scale bars were created with the LAS AF software version 1.6.0 build 1016 (Leica Microsystems, Germany).

**Ni-NTA Pull-down Assay:**

H1299 cells or MEFs were plated in 100 mm dishes and transfected with 4 µg His$_6$-ubiquitin (His-Ub) plasmid, 4 µg FOXO vectors and 4 µg MDM2 vectors using Lipofectamine Plus; 24 h post transfection, cells were harvested and separated into two aliquots. One aliquot (10%) was subjected to immunoblotting analysis to detect the expression of transfected proteins. The other aliquot of cells (90%) was used to purify the proteins of interested using Ni$^{2+}$-nitrilotriacetic acid (NTA) beads. Cell pellets were lysed in a buffer containing 0.01 M Tris-Cl (pH 8.0), 6 M guanidinium-HCl, 0.1 M sodium phosphate, 5 mM imidazole, 10 mM β-mercaptoethanol and incubated with Ni$^{2+}$-NTA beads (Qiagen) for overnight at room temperature. The beads were washed sequentially with the lysis buffer, a buffer containing 0.01 M Tris-Cl (pH 8.0), 8 M urea, 0.1 M sodium phosphate, 10 mM β-mercaptoethanol, and a buffer containing 0.01 M Tris-Cl (pH 6.3), 8 M urea, 0.1 M sodium phosphate, 10 mM β-mercaptoethanol. Proteins bound to the beads were eluted with a buffer containing 0.15 M Tris-Cl (pH 6.7), 5% SDS, 200 mM imidazole, 30% glycerol, 0.72 M β-mercaptoethanol and were subjected to immunoblotting analysis for the presence of Ub-conjugated FOXO proteins.
**In vitro Transcription Coupled Translations and GST (Glutathione S-transferase)**

**Pull-down assays:**

FOXO1 protein was produced with pcDNA3-Flag-FOXO1 as a template using T7 polymerase-based *in vitro* transcription coupled translations (Promega, Madison, Wis.). GST-MDM2 plasmids were transformed into BL21 and cultured at 37°C until the optical density at 600 nm reached 0.6. Then, 0.2 mM of isopropylthiogalactopyranoside (IPTG) was added and the incubation continued for another 5 h at 30°C. Bacterial cultures were lysed by sonication in a buffer containing 50 mM Tris (pH 8.0), 10 mM NaCl, 1 mM EDTA, 6 mM MgCl₂, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. GST pull down analyses were performed using the MagneGST pull-down system (Promega Madison, Wis) following the vendor’s protocols.

**In Vitro Ubiquitination Assays:**

Full length GST-MDM2 and GST-MDM2-NT (1-150) were expressed in *E. coli* and bound to glutathione agarose beads. The substrate FOXO1 was produced by *in vitro* transcription coupled translation in rabbit reticulocyte lysate using the TNT system (Promega) in the presence of ³⁵S- methionine. 4 µg GST-fusion proteins and 8 µl FOXO1 *in vitro* translation product were incubated to allow the enzyme-substrate interaction to occur. After three times washes with PBS containing 0.2% NP-40, the bead-bound enzyme-substrate complex was incubated at 37°C for 1 h with 250 ng GST-Ubc5Hb (Boston Biochem), 250 ng purified rabbit E1 (Boston Biochem), 2 µg His₆-Ub (Boston Biochem) in 20 µl reaction buffer containing 50 mM Tris (pH 7.5), 2.5 mM MgCl₂, 15 mM KCl, 1 mM dithiothreitol, 0.01% Triton X-100, 1% glycerol and 8 mM ATP. The reactions were terminated by boiling in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Gel was dried and the Ub-conjugated FOXO1 proteins were detected by autoradiography.
Apoptotic Analysis and Flow Cytometry:

The determination of the survival and apoptotic index of GFP (green fluorescent protein)-transfected cells has been described (Li et al., 2001). In brief, transfected cells were washed with PBS, fixed in 4% formaldehyde and stained with DAPI. Representative micrographs were captured by a charge-coupled device camera with a Smart Capture Program (Vysis, Downers Grove, Ill.) attached to a Leitz Orthoplan 2 fluorescence microscope. The viability of transfected cells in each well was determined by counting the total number of green cells in each well. The apoptotic index of GFP-positive cells was determined by scoring 300 GFP-positive cells for chromatin condensation and apoptotic body formation.

To assay the apoptosis index induced by FOXO1, H1299 cells in 100 mm dishes were transfected with GFP and FOXO1 with or without MDM2. Transfected cells were collected in PBS containing 2.5 mM EDTA, washed twice with cold PBS, re-suspended in 1× binding buffer containing 0.01 M Hepes (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl₂ at a concentration of 1×10^6 cells/ml and stained with Annexin-V APC and 7-AAD. Cell sorting and flow cytometry analysis were performed on a FAC Scan (Becton Dickinson, Mountain View, Calif.).
RESULTS

FOXO proteins are ubiquitinated and their expression level is regulated by proteasome mediated degradation (Aoki et al., 2004; Plas and Thompson, 2003), but a general ubiquitin E3 ligase for FOXO proteins remains to be identified.

Skp2 was reported to mediate the ubiquitination and degradation of FOXO1 but such activity appears to be restricted to FOXO1 (Huang et al., 2005). So far, no mutational analysis and in vitro enzyme assays have been performed to show the direct involvement of Skp2 E3 activity in FOXO ubiquitination. It remains to be determined whether Skp2 promotes FOXO1 ubiquitination directly, through its intrinsic E3 activity, or indirectly through another E3. The present study showed that MDM2 is the E3 ubiquitin ligase for FOXO factors. MDM2 interacts with FOXO, promotes the degradation and affects downstream targets and the biological function of FOXO.

1. MDM2 promotes the degradation of FOXO factors.

In an effort to investigate the interaction between p53 and FOXO signaling pathways, an inverse correlation was noticed between the expression of FOXO3A and the p53 target gene MDM2 using a panel of human cancer cell lines including osteosarcoma (Saos2), prostate (LNCaP, PC3, DU145, and JCA1), breast (MCF-7), cervical (Hela) and lung (H1299, H1299/V138) cancer cells (Figure 6). To determine whether the inverse correlation of these two proteins among cancer cells reflects a negative effect of MDM2 on FOXO protein, the level of FOXO3A and FOXO1 protein expression in p53 null and p53/MDM2 double null MEFs was measured by
immunoblotting. As shown in Figure 7, the knockout of MDM2 in MEFs resulted in an increased expression of endogenous FOXO1 and FOXO3A proteins. Consistent with the data from MEFs, the stable expression of MDM2 in H1299 cells reduced (Figure 8) and the knockdown of endogenous MDM2 increased (Figure 9) the expression of endogenous FOXO3A. Unlike MEFs, H1299/V138 cells express relatively low levels of endogenous FOXO1, which is difficult to detect. Thus, we tested the effect of MDM2 silencing on the expression of ectopic FOXO1. As shown in Figure 10, the knockdown of MDM2 by shRNA increased the level of ectopic Flag-FOXO1 expression.

Because MDM2 is an E3 ubiquitin ligase that promotes proteasome mediated degradation of p53 (Honda et al., 1997) and other (Lin et al., 2002a) proteins, it is likely that MDM2 decreases the expression of FOXO factors in a proteasome-dependent manner. In transient transfection studies, co-expression of MDM2 reduced the expression level of Flag-FOXO1 in DU145 prostate cancer cells, a process that was prevented by treatment with two different concentrations of the proteasome inhibitor MG132 (Figure 11).

To test whether the decreased FOXO protein expression by MDM2 is due to protein degradation, the half-life of Flag-FOXO1 was measured in H1299 cells cotransfected with control vector, full length MDM2 or an MDM2 (1-361) mutant which lacks the C-terminal E3 ligase domain. As shown in Figure 12, the half life of FOXO1 was decreased by full length MDM2 from 6 h to 3 h, as compared to the control vector. When FOXO1 was cotransfection of MDM2 (1-361), MDM2 (1-361) significantly extended the half-life of Flag FOXO1; the exact meaning of the latter is unclear. Overall, the data raise the possibility that MDM2 may function as an E3 ubiquitin ligase for mammalian FOXO factors.
Figure 6  Inverse correlation of MDM2 and FOXO3A protein expression in different cancer cell lines. DU145, JCA, H1299-V138, HeLa, LNCaP, MCF7, PC3, Saos-2, H1299 cells were plated into 100 mm dishes. MDM2 and FOXO3A proteins were detected by Western blotting with anti-MDM2 (SMP-14) and anti-FOXO3A (H-144) antibodies, respectively.
Figure 7  Inverse correlation of MDM2 and FOXO protein expression in MEFs. p53-/- MEFs and p53-/-, MDM2-/- double null MEFs were plated in 100 mm dishes. The cells were harvested the next day and cell lysates were subjected to immunoblotting analyses with anti-MDM2 (SMP-14) antibody, anti-FOXO1 antibody, and anti-FOXO3A (H-144) antibodies, respectively. The β-actin level showed the equal loading.
Figure 8  Stably expressed MDM2 decreases the level of endogenous FOXO3A expression. H1299 cells stably expressing MDM2 (MDM2-1, MDM2-2) or empty vector (Control) were established and cell extracts were subjected to Western blot analyses with the indicated antibodies.
Figure 9  Knockdown of endogenous MDM2 by siRNA causes an increase in endogenous FOXO3A protein. H1299 cells were transfected with control GFP-siRNAs or siRNAs (MDM2-siRNA1, MDM2-siRNA2) for MDM2, and cell extracts were subjected to Western blot analyses.
Figure 10  Knockdown of endogenous MDM2 by siRNA increases the level of ectopic FOXO1 protein. A Flag-tagged FOXO1 expression vector was cotransfected transiently with MDM2-siRNA or control siRNA (GFP-siRNA) into H1299-V138 cells, and whole cell extracts were prepared and subjected to Western blot analyses.
Figure 11  MDM2 overexpression causes a decrease in Flag-FOXO1 protein, which is blocked by MG132. DU145 cells were transfected with Flag-FOXO1 and pcMDM2. 24 h posttransfections, cells were incubated with or without different concentrations of MG132 for 6 h. Cell lysates were separated in a 8% SDS-PAGE. FOXO1 and MDM2 were detected by Western blotting with anti-Flag (M2) and anti-MDM2 (SMP-14) antibodies, respectively. Equal amounts of protein were loaded.
Figure 12  MDM2 Overexpression results in a decrease in half-life of the FOXO1 protein. H1299 cells were transfected with the plasmids as indicated. 24 h after transfection, cells were treated with CHX for different times. The cell lysates were separated by SDS–8% PAGE. Exogenous FOXO1 was detected by Western blotting with anti-Flag M2 antibodies. Equal amounts of protein were loaded on each sample.
2. **MDM2 interacts with FOXO factors *in vivo* and *in vitro.*

Ubiquitin E3 ligases are known to make direct contact with their substrates. Therefore, it was investigated whether or not MDM2 and FOXO factors form a complex in cells. Deconvolution imaging analysis detected ectopic MDM2 and FOXO1 proteins in both cytoplasmic and nuclear compartments of p53/MDM2 double null MEFs, but colocalization was detected predominantly in the nucleus (Figure 13). Similarly, confocal imaging gave the same result for endogenous MDM2 and FOXO3A in H1299 cells (Figure 14).

To determine whether MDM2 and FOXO1 interact in mammalian cells, they were ectopically expressed in H1299 cells and reciprocal coimmunoprecipitations performed. MDM2 and FOXO1 were co-precipitated in cells expressing both proteins. In cells that express either MDM2 or FOXO1, little or no coprecipitations were observed, suggesting that co-precipitations were not due to cross reactivity of the antibodies (Figure 15). Similar analysis showed that MDM2 and GFP-FOXO3A were specifically co-precipitated, but not MDM2 and GFP (Figure 16).

To determine whether endogenous MDM2 and FOXO1 interacted in mammalian cells, H1299/V138 cells expressing a temperature-sensitive p53 mutant (Pochampally et al., 1999) were shifted to permissive temperature for 16 h to induce MDM2 expression and cellular extracts were subjected to coimmunoprecipitation analysis. As shown in Figure 17, FOXO1 was co-precipitated by the anti-MDM2 antibody, but not by the control antibody, showing that the co-precipitations were not due to antibody cross reactivity.

To determine whether endogenous MDM2 and FOXO3A form a complex, extracts of HEK293T cells were treated with DMSO or MG132 and co-immunoprecipitations with an anti-MDM2 antibody. An anti-HA antibody was
Figure 13  Exogenous MDM2 and Flag-FOXO1 are colocalized in the nucleus of MEF cells. p53-/-, MDM2-/- MEF in a medium containing 0.5% FBS were transfected with 1 μg pcMDM2 and 1 μg Flag-FOXO1. Cells were stained with DAPI and incubated with anti-MDM2 (SMP14) and anti-FOXO1 (H-128) antibodies. Immunoreactivity was detected with IgG conjugated to Alexa Fluor 594 (red for MDM2) or FITC (green for FOXO1). Colocalization was determined by high-resolution imaging with deconvolution microscopy.
Figure 14  Endogenous MDM2 and FOXO3A are colocalized in the nucleus of H1299 cells. H1299 cells in a medium containing 1% FBS were stained with DAPI and incubated with anti-MDM2 (SMP14) and anti-FOXO3A (H-144) antibodies. Immunoreactivity was detected with IgG conjugated to Alexa Fluor 594 (red for MDM2) or FITC (green for FOXO3A). Colocalization was visualized by high-resolution imaging with confocal microscopy.
**Figure 15** Interaction of ectopic FOXO1 and MDM2 in H1299 cells.

H1299 cells were cotransfected with HA-tagged FOXO1 and pcMDM2. 24 h posttransfection, cell extracts were immunoprecipitated with either anti-HA or anti-MDM2 antibodies followed by immunoblotting with anti-MDM2 or anti-HA antibody.
**Figure 16** Interaction of ectopic FOXO3A and MDM2 in HEK293T cells.

HEK293T cells were cotransfected with pcMDM2 and either GFP or GFP-FOXO3A. 24 h posttransfection, cell extracts were immunoprecipitated with either an anti-MDM2 or an anti-GFP antibody followed by immunoblotting with an anti-GFP or an anti-MDM2 antibody.
Figure 17 Interaction of endogenous MDM2 and FOXO1 in H1299/V138 cells. Cells were shifted from 39°C and cultured in 33°C for overnight and were treated with or without MG132 for 6 h. Cell extracts were immunoprecipitated with an anti-FOXO1 antibody followed by immunoblotting with an anti-MDM2 antibody.
Figure 18  Interaction of endogenous MDM2 and FOXO3A in HEK293T cells. HEK 293T cells were treated with or without MG132 for 6 h before harvest. Cell extracts were immunoprecipitated with either an anti-HA antibody as control or an anti-MDM2 (SMP14) antibody followed by immunoblotting with an anti-FOXO3A antibody.
Figure 19  The interaction between FOXO1 and GST-MDM2 in vitro.

FOXO1 synthesized by in vitro transcription-translation reactions was incubated with GST-MDM2 fusion proteins, precipitated with glutathione beads and detected by immunoblotting with an anti-FOXO1 antibody. The amounts of GST proteins used in the pull-down assays were visualized by Coomassie blue staining after separation in a SDS-PAGE gel (bottom panel).
included as a control. As shown in Figure 18, FOXO3A proteins were co-precipitated with MDM2 by an anti-MDM2 but not an anti-HA antibody. Treatment with MG132 increased the level of MDM2 and FOXO expression as well as the amount of proteins co-precipitated. These studies showed that co-precipitations occur with both endogenous and ectopically expressed proteins.

To determine whether MDM2 and FOXO1 interact in vitro, GST and GST-MDM2 fusion proteins were produced in bacteria, bound to glutathione beads and incubated with FOXO1 proteins produced by in vitro transcription coupled translations. In these GST pull down assays, wild type FOXO1 was precipitated with GST-MDM2, but not with GST (Figure 19). An active form of FOXO1, FOXO1 (AAA) in which all three AKT phosphorylation sites were mutated to alanine, was also precipitated with GST-MDM2 but not with GST (Figure 19). This analysis demonstrated that FOXO1 and MDM2 form a complex in vitro and that complex formation occurs independently of the phosphorylation of FOXO1 by AKT.

3. The fork head box of FOXO and the region of MDM2 controlling nuclear-cytoplasmic shuttling mediate the interaction between MDM2 and FOXO.

To define the region in FOXO1 that mediates the interaction with MDM2, H1299 cells were transfected with full length MDM2 and FOXO1 deletion constructs fused to the HA tag (Figure 20a). Cellular extracts were subjected to co-immunoprecipitation with an anti-MDM2 antibody. As shown in Figure 21, the full length FOXO1 protein was co-precipitated with MDM2. Deletion of the C-terminal region of FOXO1 did not alter the interaction whereas further deletion into fork head box abolished it, suggesting that the fork head box is required for the interaction.

Further define the region in FOXO3A that mediates the interaction with MDM2, purified bacteria expressed GST or GST fused fragments of FOXO3A (peptide P1-P5)
encoding five nonoverlapping FOXO3A regions (Figure 20b) were incubated with HEK293T cell extracts overexpressed MDM2. MDM2 coprecipitated only with peptide P2 (amino acid 154-259), which contains the forkhead domain (Figure 22). These results suggest that MDM2 specifically interacts with the forkhead domain of FOXO3A. Further alignment of the forkhead domain of mouse FOXO3A, human FOXO1, FOXO3A and FOXO4 (Figure 23), we found that the forkhead domain is highly conserved in these four kinds of FOXO. All these data indicated MDM2 has a general effect on FOXO.

To define the region of MDM2 responsible for FOXO1 binding, different MDM2 constructs (Figure 24) were transfected into H1299 cells together with Flag-FOXO1. Cellular extracts were immunoprecipitated with MDM2 antibody and the co-precipitated FOXO1 was detected by anti-Flag antibody. As shown in Figure 25, all MDM2 mutants, except MDM2 (50-491) (p53 binding deficient) and MDM2 NLS-mt (182R, nuclear localization sequence defective), interacted with FOXO1. Immunoblot analysis showed that all MDM2 mutants were expressed at significant levels and most MDM2 proteins were detected in multiple forms, presumably due to cleavage by proteases (Chen et al., 1997). In the presence of proteasome inhibitor MG132, however, both MDM2 (50-491) and MDM2 NLS-mt were co-precipitated with Flag-FOXO1 by the anti-Flag antibody (Figure 26). The exact reason for the lack of interaction with FOXO1 in the absence of MG132 is unclear, presumably due to the degradation of the protein complex by the proteasome.

Further analysis with additional MDM2 mutants in the presence of MG132, showed that the deletion of 150-230 amino acids abrogated the FOXO1 binding (Figure 27). Consistent with the fact that this region contains the nuclear localization sequence, it was also shown (Figure 28) that this mutant is mainly localized to cytoplasm. However, cytoplasmic localization is clearly not the reason for the lack of interaction with MDM2.
Figure 20  Diagram of different FOXO mutants used in this study.

a, The different mutants of human FOXO1 used in this study; b, The different mutants of mouse FOXO3A used in this study.
Figure 21  Mapping of the MDM2-interacting domain of FOXO1 by immunoprecipitation. H1299 cells were transfected with 2 µg MDM2 and 2 µg of different HA-tagged FOXO1 fragments. Anti-DM2 immunoprecipitates were subjected to immunoblotting with anti-HA antibody and anti-MDM2 as indicated. Immunoblotting of total cell extracts with anti-HA antibody (lower panel) showed the expression of different FOXO1 fragments.
Figure 22  Mapping of the MDM2-interacting domain of FOXO3A by GST pull-down assay. Recombinant GST or GST fused with fragments of FOXO3A (P1-P5) were amplified by bacteria expression and purified by GST beads. HEK 293T cells were transfected with 4 µg MDM2. Cell lysates were subjected to GST pull down experiment. The amounts of GST proteins used in the pull-down assays were visualized by Coomassie blue (CB) staining after separation in a SDS-PAGE gel (bottom panel).
Figure 23  Alignment of FOXO member's fork head box. Grey box – fork head box; Green box – unique FOXO sequence.
Figure 24  Diagram of different MDM2 mutants used in the study.
Figure 25  Mapping of the FOXO1-interacting domain in MDM2 by coimmunoprecipitations in the absence of MG132. H1299 cells were transfected with 2 µg Flag-FOXO1 and 2.5 µg different MDM2 fragments. Anti-MDM2 immunoprecipitates were probed with anti-Flag M2 antibody (upper panel). Immunoblotting of cellular extracts with anti-MDM2 antibody (lower panel) showed the expression of different MDM2 fragments.
Figure 26 An N-terminal truncation mutant and an NLS-mutant of MDM2 interact with FOXO1 in the presence of MG132. H1299 cells were transfected with 2 µg Flag-FOXO1 together with 5 µg MDM2 or 5 µg mutant MDM2. Cells were treated with MG132 for 6 h before harvest. Cells lysate were immunoprecipitated with anti-Flag M2 antibody and detected with anti-MDM2 antibody. FL: full length MDM2, 50-491; MDM2(50-491); NLS: MDM2(NLS-mt).
Figure 27  Truncation of the central region of MDM2 abolishes the interaction between MDM2 and FOXO1. H1299 cells were transfected with 2 µg Flag-FOXO1 and 2.5 µg of different MDM2 fragments. Cells were treated with MG132 for 6 h before harvesting. Anti-Flag immunoprecipitates were probed with anti-MDM2 (2A10) antibody (upper panel). Immunoblotting of cellular extracts with anti-MDM2 antibody (lower panel) showed the expression of different MDM2 fragments.
Figure 28  Immunofluorescence images show the cellular localization of various MDM2 mutants. H1299 cells were transiently transfected with MDM2 or different MDM2 mutants. 24 h posttransfection, cells were fixed and stained. The red signal showed the localization of MDM2 and the blue signal showed the nucleus of the cells.
because co-precipitations were done with whole cell extracts and, under the same conditions, nuclear localization sequence mutant, the MDM2 NLS-mt, interacted with FOXO1 (Figure 26). Besides cellular localization sequences, the 150-230 region also contains an inhibitory domain that suppresses cell cycle progression independently of p53. This region is also involved in interactions with several proteins, including TBP and p300.

4. MDM2 promotes the ubiquitination of FOXO1 and FOXO3A.

To test whether MDM2 promoted the ubiquitination of FOXO factors, H1299 cells were transfected with Flag-tagged FOXO1, wild type or an AKT phosphorylation site FOXO1 mutant. The effect of ectopic MDM2 on FOXO1 ubiquitination by cotransfected Myc-tagged ubiquitin was measured in the anti-Flag precipitates. In the absence of ectopic MDM2, limited FOXO1 ubiquitination was occurred. This result is consistent with the fact that H1299 cells are p53-deficient and contain low levels of endogenous MDM2. Cotransfection of MDM2 with increased the level of ubiquitilation of the wild type FOXO1 but not of FOXO1 (AAA). The data argue that the positive effect of MDM2 on FOXO1 ubiquitination requires phosphorylation at the AKT sites (Figure 29).

To test whether the effect of MDM2 extends to other FOXO factors, FOXO3A and His-tagged ubiquitin were transfected into H1299 cells and the effect of MDM2 on FOXO3A ubiquitination was measured by immunoblotting with anti-HA antibody following nickel bead pull-down under denaturing conditions. As shown in Figure 30, FOXO3A ubiquitination was increased by MDM2 in a manner dependent on the phosphorylation on the AKT sites. The data suggest that the stimulation of ubiquitination by MDM2 is not restricted to FOXO1 but among the FOXO factors.

In p53 and MDM2 double-null MEFs, wild type MDM2 stimulated the ubiquitination of FOXO1 in a dose dependent manner, whereas the MDM2 mutant
lacking the C-terminal ring finger E3 region did not exert such an effect (Figure 31), emphasizing the potential involvement of the E3 ligase activity. Nickel bead pull down assays under denaturing conditions revealed that MDM2 containing a point mutation in the E3 ligase domain, MDM2 E3-mt (457S), did not stimulate FOXO1 ubiquitination (Figure 32), confirming that FOXO1 ubiquitination by MDM2 requires its ubiquitin ligase activity. Interestingly, on the one hand, a constitutively nuclear MDM2 in which the nuclear export sequence was mutated, the NES-mt, did not promote FOXO1 ubiquitination (Figure 33), even though it contained an intact E3 ligase domain and interacted with FOXO1 (Figure 25). On the other hand, two cytoplasmic MDM2 mutants, MDM2 NLS-mt and MDM2 (Δ89-150), stimulated FOXO1 ubiquitination (Figure 32 and 33), showing that the ubiquitination of FOXO1 by MDM2 is likely to occur in the cytoplasm. MDM2 (Δ150-230), which was located mainly in the cytoplasm (Figure 25) and did not interact with FOXO1 (Figure 26), was unable to stimulate FOXO1 ubiquitination, suggesting that the ubiquitination requires FOXO1 interaction. Overall, the data suggest that FOXO1 interacts with MDM2 in both the nucleus and the cytoplasm, but the ubiquitination by MDM2 requires the interaction in the cytoplasm.

To fully establish MDM2 as an E3 ligase for FOXO1 ubiquitination, the ability of recombinant MDM2 to catalyze the ubiquitination of FOXO1 was tested in vitro. In this experiment, GST-MDM2 stimulated the ubiquitination of in vitro translated FOXO1 in the presence but not in the absence of purified E1, E2 and ubiquitin (Figure 34, upper panel). GST fused to an MDM2 N-terminal fragment had no effect on the ubiquitination of FOXO1. In reactions performed with transcription-coupled translation product from a control vector, the majority of the 35S-labeled ubiquitin conjugates disappeared, showing that they were FOXO1 proteins (Figure 34, lower panel). In combination with the binding
**Figure 29**  MDM2 promotes the ubiquitination of FOXO1. H1299 cells were transfected with the indicated plasmids and cell extracts were either immunoprecipitated with M2 antibody, followed by immunoblotting with anti-Myc antibody, or directly immunoblotted with antibodies to Flag or MDM2.
Figure 30  MDM2 promotes the ubiquitination of FOXO3A. H1299 cells were transfected with the indicated plasmids and treated with MG132 for 6 h before harvest. Cell lysates were subjected to pull down analyses with Ni-NTA beads followed by immunoblotting with anti-FOXO3A antibody. Cell extracts were also subjected to direct immunoblotting by anti-MDM2 antibody.
Figure 31  The MDM2 ring finger domain is critical for the ubiquination of FOXO1. p53-/-,MDM2-/--MEF cells were transfected with the indicated plasmids including Myc-tagged ubiquitin and cell extracts were either immunoprecipitated with M2 antibody followed by immunoblotting with an anti-Myc antibody or immunoblotted directly with antibodies to Flag or MDM2.
Figure 32  Ubiquitination of FOXO1 requires the MDM2 ubiquitin ligase function. P53−/−, MDM2−/− MEFs were transfected with Flag-FOXO1, His-Ubiquitin and MDM2 mutants. Cell extracts were subjected to pull down assays with Ni-NTA beads followed by immunoblotting with anti-FOXO1 antibody.
Figure 33  The central region of MDM2 cannot promote the polyubiquitination of FOXO1. H1299 cells were transfected with 2 μg Flag-FOXO1, 4 μg MDM2, and 4 μg His-ubiquitin. Cell extracts were subjected to pull down analyses with Ni-NTA beads followed by immunoblotting with anti-FOXO1 antibody.
Figure 34  MDM2 promotes FOXO1 polyubiquitination \textit{in vitro}. GST-MDM2 and GST-N (containing MDM2 residues 1 to 150) were purified using glutathione agarose beads. Loaded beads were incubated with in vitro-translated FOXO1 in the presence or absence of E1 and E2 in an ubiquitination reaction as described in Materials and Methods. Polyubiquitinated FOXO1 appears as a high molecular weight smear above the unmodified FOXO1 band.
data and whole-cell ubiquitination analysis with MDM2 mutants, the *in vitro* data established that MDM2 functions as an ubiquitin E3 ligase for FOXO proteins.

5. **MDM2 suppresses the expression of FOXO target genes and protects cells from FOXO1-induced cell death.**

FOXO target genes tumor necrosis related apoptosis inducing ligand (TRAIL), p27 CDK inhibitor and manganese superoxide dismutase (MnSOD) mediate the effect of FOXO proteins on cell cycle arrest, apoptosis, and detoxification of reactive oxygen species. Their transcription products are directly regulated by FOXO factors. Consistent with the ubiquitination and degradation of FOXO factors by MDM2, stable expression of MDM2 in H1299 cells decreased the level of TRAIL, p27 and MnSOD expression (Figure 35) whereas the knockdown of MDM2 increased the expression of TRAIL (Figure 36).

To test whether MDM2 protects cells from FOXO induced cell death, H1299 cells were transiently transfected with GFP and either control vector, FOXO1 or FOXO1 in combination with MDM2. The survival of the transfected cells was analyzed. The expression of FOXO1 decreased the number of transfected (green) cells, an effect that is relieved by MDM2 co-expression (Figure 37a). To confirm that the change in the viability of transfected cells is the result of cell apoptosis, transfected cells were fixed and stained with DAPI and their nuclear morphology was examined for features of apoptosis under a fluorescence microscope that allows the simultaneous visualization of blue and green fluorescence. Apoptotic index, as determined by scoring apoptotic cells in 300 green cells per sample, was 5% for controls and 25% for cells transfected with FOXO1. Co-expression of MDM2 suppressed FOXO1-induced cell death in a dose-dependent manner (Figure 37b). The data were reproduced by independent
Figure 35    Stable expressed MDM2 in H1299 cells regulates the expression of downstream targets of FOXO. Extracts of control (H1299-VEC) and MDM2 stable (H1299-M1) clones were subjected to immunoblotting with different antibodies as indicated in the figure.
**Figure 36**  MDM2 siRNA increases the expression of the FOXO1 target gene TRAIL. H1299 cells were transiently transfected with either GFP-siRNA or MDM2-siRNA. The cells were harvested at 48 h later. Cell extracts were subjected to immunoblotting analyses with anti-MDM2, anti-FOXO3A and anti-TRAIL antibodies.
Figure 37  MDM2 promotes the cell survival in the presence of FOXO1.
Figure 37  MDM2 promotes the cell survival in the presence of FOXO1.
a, H1299 cells were transfected with pLNCE and Flag-FOXO1 in the presence or absence of MDM2. The viability of transfected cells in each well was scored by counting the number of green cells. Representative micrographs were captured by the fluorescence microscope that had a charge-coupled device camera. b, H1299 cells were transfected with the same plasmids as in a, Apoptotic index of GFP-positive cells was determined by scoring 300 GFP-positive cells for chromatin condensation and nuclear fragmentation. Triplicate samples were analyzed per data point, and the graph represents three independent experiments.
Figure 38  MDM2 protects cells from FOXO1-induced cell death measured by Flow Cytometry.
Figure 38  MDM2 protects cells from FOXO1-induced cell death measured by Flow Cytometry. H1299 cells were transfected with GFP-spectrin and Flag-FOXO1 in the presence or absence of MDM2. The apoptotic GFP positive cells were detected with Annexin-V APC and 7-AAD. a, bar graphs; b, representative flow cytometry profiles.
analysis of early apoptosis with the Annexin V method after FACS-based sorting. As shown in Figure 36, two independent analyses of cells cotransfected with GFP-spectrin showed that FOXO1 expression increased apoptosis in transfected cells by about 3-fold, which is partially suppressed by the co-tranfection of MDM2. The degree of induction by FOXO1 and the suppression by MDM2 varied between two experiments because the basal line of the FACS machine varied from time to time.

6. MDM2 transiently increased FOXO transcriptional activity.

Since FOXO factors are transcriptional factors, an important question which must be addressed is whether or not MDM2 affects the FOXO transcriptional activity. In order to answer this question, the following reporter genes were used in our experiment. Synthetic reporter $3 \times$ IRSLuc which contained three conserved insulin response sequence (IRS). FOXO1, like insulin, promotes the promoter activity through an IRS. Cyclin D is the downstream target of FOXO factors in the cell cycle check point. FOXO factors expression results in reduced levels of cyclin D protein expression. Cyclin D-Luc is the reporter gene which can be used to specifically measure cyclin D activity in cells. In order to measure the activity of the reporter gene, a transient transfection was performed by transfecting the reporter gene, CMV-gal as an internal control, pcDNA3 control or Flag-FOXO1, and different doses of MDM2 into the DU145 cells and NIH3T3 cells. The data showed that MDM2 increased the $3 \times$ IRSLuc activity, and this increase occurred in a dose dependent manner (Figure 39 & Figure 40). FOXO1 decreased the cyclin D-Luc activity and MDM2 decreased it further in a dose dependent manner (Figure 41).

Our earlier data revealed that MDM2 interacted with both wild-type FOXO1 and FOXO1 (AAA), however it only promoted the polyubiquitination of wild-type FOXO1.
Figure 39  MDM2 increases the transcriptional activity of FOXO1 in a dose-dependent manner in DU145 cells. DU145 cells were transfected with 0.5 μg 3xIRSLuc, 0.1 μg CMV-Gal, 0.1 μg control vector or Flag-FOXO1, and different amounts of MDM2. FOXO activity was measured by the Promega luciferase activity kit.
Figure 40  MDM2 increases the transcriptional activity of FOXO1 in NIH3T3 cells. NIH3T3 cells were transfected with 0.5 μg 3×IRSLuc, 0.1 μg CMV-Gal, 0.1 μg Flag-FOXO1, and different amounts of MDM2. FOXO activity was measured by the Promega luciferase activity kit.
Figure 41  MDM2 enhances the ability of FOXO1 to inhibit cyclin D1.  
H1299 cells were transfected with 0.5 μg cyclin D-Luc, 0.1 μg CMV-Gal, 0.1 μg Flag-FOXO1 and different amounts of MDM2. FOXO activity was measured by the Promega luciferase activity kit.
Figure 42  MDM2 increases the transcriptional activity of both wild type FOXO1 and the FOXO1(AAA) mutant in NIH3T3 and DU145 cells. Cells were transfected with 0.5 μg 3×IRSLuc, 0.1 μg CMV-gal, 0.1 μg Flag-FOXO1 or FOXO1 (AAA) and different amounts of MDM2. FOXO activity was measured by the Promega luciferase activity kit. a, DU145; b, NIH3T3.
MDM2 also increased the transcriptional activity of FOXO1 (AAA) (Figure 42). The data further indicated that MDM2 promotes FOXO transcriptional activity in an AKT-independent fashion.

7. p53 induces transient increase in the transcriptional activity of FOXO factors, which is followed by FOXO degradation in an MDM2-dependent manner.

To test whether p53 affects the transcriptional activity of FOXO1, reporter gene 3 × IRSLuc, CMV-gal, pcDNA3 control or Flag-FOXO1 or Flag-FOXO1 (AAA), and different doses of p53 were transfected into DU145 cells (contain endogenous mutated p53) and LNCaP cells (contain endogenous wild type p53) and the transcriptional activity of FOXO1 was determined. p53 decreased FOXO1 transcriptional activity in both cell lines (Figure 43a & Figure 44), but did not inhibit FOXO1-induced decrease in cell viability (Figure 43b). Introduction of MDM2 into the cells relieved the inhibition of FOXO activity by p53 (Figure 45).

In order to better understand the interaction among FOXO factors, p53 and MDM2, H1299/V138 stable cell line that expresses a temperature-sensitive p53 was used. Shifting to a permissive temperature allows the activation of p53. At different time points after p53 activation, the transcriptional activity of endogenous FOXO factors and the level of FOXO3A and MDM2 protein expression were determined. Reporter analyses showed that FOXO activity was transiently induced between 5 and 24 hours after p53 activation and subsequently decreased (Figure 46a). Immunoblotting analysis showed that the MDM2 protein was induced to the highest level 5 hours after p53 activation followed by gradually decrease of FOXO3A and MDM2 level (Figure 46b). MG132 treatment prevented the time-dependent decrease in FOXO3A levels (Figure 47), suggesting that the decrease is due to proteasome-mediated degradation. In order to
Figure 43  P53 inhibits the transcriptional activity of FOXO in a dose-dependent manner in DU145 cells, but not FOXO1-induced cell death. a, DU145 cells were transfected with 0.5 μg 3xIRSLuc, 0.1 μg CMV-Gal, 0.1 μg Flag-FOXO1 and different amounts of p53. FOXO activity was measured by the Promega luciferase activity kit. b, Cells were transfected with pLNCE and plasmids as described in panel a. Representative micrographs were captured by the fluorescence microscope that had a charge-coupled device camera.
Figure 44  P53 inhibits the transcriptional activity of FOXO in a dose-dependent manner in LNCaP cells. DU145 cells were transfected with 0.5 μg 3×IRSLuc, 0.1 μg CMV-Gal, 0.1 μg Flag-FOXO1 and different amounts of p53. FOXO activity was measured by the Promega luciferase activity kit.
Figure 45  MDM2 relieves the repression of FOXO1 activity by p53.

DU145 cells were transfected with 0.5 μg 3xIRSLuc, 0.1 μg CMV-gal, 0.1 μg Flag-FOXO1, 0.1 μg HA-p53 and 0.5 μg of either control vector or MDM2. FOXO activity was measured by luciferase activity kit (Promega).
Figure 46   MDM2 transiently increases FOXO transcriptional activity, which is followed by FOXO degradation. H1299/V138 cells cultured at 39°C were shifted to 32°C for the indicated length of time. Cell extracts were prepared and assayed by luciferase assay (panel a) or by immunoblotting analyses (panel b).
**Figure 47** MG132 relieves p53-induced decrease in the expression of FOXO3A protein in H1299/V138 cells. Cells cultured at 39°C were shifted to 32°C for indicated length of time. Cells were treated with MG132 for 6 h before cell extracts were prepared and subjected to immunoblot analysis.
Figure 48  p53 decreases the protein level of FOXO1 through MDM2.

H1299 cells were transiently transfected as noted on the Figure. 24h after transfection, cell lysates were subjected to immunoblotting with M2 antibody (to detect FOXO1 expression), anti-HA antibody (to detect p53 expression), and anti-MDM2 (SMP14). Tubulin blots was included to show even loading in each sample.
**Figure 49** Knockdown of MDM2 partially relieves p53-induced FOXO3A downregulation. H1299/V138 cells were transfected with scrambled or MDM2 siRNA. 24 h posttransfection, cells were shifted to 32°C and cultured for another 18 h. Then immunoblotting was performed with the indicated antibodies.
determine which protein led to the degradation of FOXO factors, a transient transfection experiment was performed in H1299 cells. Immunoblotting analyses showed that p53 did not decrease the level of FOXO1 protein expression. MDM2, either alone or in combination with p53, decreased the level of FOXO1 protein expression (Figure 48). Knockdown of MDM2 by siRNA partially relieved FOXO3A down-regulation by active p53, supporting the conclusion that the p53-induced decrease is MDM2-dependent (Figure 49).

8. Site-specific sumoylation of SIRT1 regulates FOXO1 transcriptional activity and stability.

Acetylation can affect protein degradation via ubiquitination (Ito et al., 2002; Jeong et al., 2002; Li et al., 2002). The study in pancreatic β cells showed that FOXO1 acetylation inhibited the degradation of FOXO1 via the ubiquitin pathway (Kitamura et al., 2005).

SIRT1 is a mammalian HDAC. As a potential nutrient sensor, it regulates the lifespan of mammals in response to caloric restriction or nutrient starvation, and protects cells from apoptosis induced by DNA damage.

Previously (Brunet et al., 2004) and our studies (Figure 50) showed that SIRT1 inhibited FOXO1 transcriptional activity. After treating the H1299/V138 cells with SIRT1 inhibitor nicotinamide, we found that nicotinamide caused further increase of FOXO1 activity from 24 hours to 48 hours post p53 activation (Figure 51). Therefore SIRT1 has a role in the action of p53-MDM2 on the activity and degradation of FOXO1.

Since SIRT1 affects the FOXO1 transcriptional activity and plays a potential role in FOXO1 stability, two very interesting questions arise: How does SIRT1 affect the transcriptional activity of FOXO1 and how is SIRT1 activity regulated. Our previous study
Figure 50  SIRT1 inhibits the transcriptional activity of FOXO. H1299 cells were transfected with 0.5 μg 3×IRS Luc, 0.1 μg CMV-Gal, 0.1 μg Flag-FOXO1 and 0.5 μg HA-SIRT1. FOXO activity was measured by the Promega luciferase activity kit.
Nicotinamide treatment increases the FOXO transcriptional activity. H1299 and H1299/V138 cells were transfected with 0.5 μg 3×IRS Luc and 0.1 μg CMV-Gal. 16 h later, the cells were treated with nicotinamide and transferred from 37°C to 32°C at indicated time points. FOXO activity was measured by the Promega luciferase activity kit.
Figure 52  SIRT1 sumoylation at Lys 734 is required for FOXO1 deacetylation. H1299 cells were co-transfected with Flag-FOXO1, HA-p300 and either wild-type HA-SIRT1 (WT) or HA-SIRT1 mutated at Lys 734 (734R). Anti-Flag M2 immunoprecipitates were immunoblotted with antibody to acetylated FOXO1 or with an antibody to FOXO1. Cellular extracts were also immunoblotted with antibody to HA.
Figure 53  Mutation of Lys 734 relieves the inhibition of FOXO1 transcriptional activity by SIRT1. The activity of transfected FOXO1 was measured using an IGFBP1 promoter-based reporter in H1299 cells expressing wild type SIRT1 or SIRT1 (734R) in the absence or presence of p300. CTL-control.
showed that the sumoylation status of SIRT1 affects its deacetylase activity since a sumoylation site specific mutation abolished the deacetylase activity of SIRT1.

FOXO1 is a known substrate of SIRT1. In order to determine whether this sumoylation site affects the effect of SIRT1 on FOXO, Flag-tagged FOXO1 was cotransfected with p300, wild-type SIRT1 or SIRT1 (734R) in which the sumoylation site of SIRT1 was mutated, acetylated FOXO1 was detected by Western blotting of M2 immunoprecipitates with an antibody that recognizes acetylated FOXO1. As shown in Figure 52, expression of wild type SIRT1 reduced amount of acetylated Flag-FOXO1 induced by p300, whereas expression of SIRT1 mutated at Lys 734 did not. When the cells transfected either FOXO1 with SIRT1, SIRT1 decreased FOXO1 transcriptional activity which is consistent with the previous study, whereas expression of SIRT1 mutated at Lys734 could not decrease the transcriptional activity of these two proteins (Figure 53). These data showed that the sumoylation status of SIRT1 is critical for its effect on FOXO1.

9. Genistein-induced FOXO1 expression is blocked by MDM2 expression in H1299 cells.

Genistein is considered the primary anticancer component of soybeans. Its in vitro and/or in vivo activities include the antagonism of estrogen, inhibition of protein tyrosine phosphorylation, suppression of angiogenesis, inhibition of hydrogen peroxide formation induced by tumor promoters, inhibition of topoisomerases, induction of apoptosis and cell differentiation, scavenging of free radicals, and inhibition of carcinogenesis and tumor promotion. A previous paper showed that genistein downregulated the MDM2 oncogene, induced apoptosis and inhibited proliferation in a variety of human cancer cell lines, regardless of p53 status(Li et al., 2005). This raised an
**Figure 54** Genistein increases the expression of FOXO through MDM2 downregulation. H1299-VEC and H1299-M1 cells were plated in 100 mm dishes and treated with indicated doses of genistein for 24 h. Cell lysates were subjected to immunoblotting with anti-FOXO1 and anti-MDM2 antibodies.
interesting question whether genistein’s anti-tumor activity is mediated through FOXO factors.

To address this question, parental H1299 cells and H1299 cells that stably express MDM2 (H1299-M) were plated in 100 mm dishes and treated with genistein at different doses. The expression of MDM2 and FOXO1 was determined by Western blot analysis. The analyses revealed that genistein decreased MDM2 and increased FOXO1 level in a dose-dependent manner in H1299 parental cells. In H1299-M cells, MDM2 expression was detected at a very high level. Genistein treatment had little effect on the expression of MDM2 or FOXO1 proteins (Figure 54). The data indicated that genistein increases FOXO1 level through MDM2 down regulation. It also indicated that FOXO factors may be one of the nuclear targets of genistein in suppressing tumorigenesis.

10. **ARF promotes the MDM2-induced FOXO ubiquitination.**

ARF is encoded by the *INK4a-ARF* locus and it is a known inhibitor of the MDM2 E3 ligase function on p53. To test whether ARF affects MDM2 E3 ligase function of FOXO factors, H1299 cells were transfected with Flag-FOXO1, MDM2, myc-ARF and His-ubiquitin as indicated in the figure 53. The cell lysates were purified by Ni-NTA beads. The ubiquitinated-FOXO1 was detected by M2 anti-Flag antibody. As shown in Figure 63, ARF increased FOXO1 ubiquitination level to a degree similar to that induced by MDM2 (Figure 55). The combination of ARF and MDM2 increased the level of FOXO1 ubiquitination to a degree comparable with that of MDM2 alone, suggesting that AR may act through MDM2 to regulate FOXO ubiquitination (Figure 55). These data are quite opposite to p53 ubiquitination induced by MDM2 but are consistent with our conclusion that FOXO ubiquitination occurs in the cytoplasm. It is known that ARF inhibits the target of MDM2 to nucleoli to suppresses the ubiquitination of p53.
Figure 55  ARF promotes the MDM2-induced FOXO1 ubiquitination. H1299 cells were transfected with 2μg Flag-FOXO1, 4 μg MDM2, 4 μg myc-ARF, 4 μg His-ubiquitin as described on the Figure. Cell extracts were pull-down by Ni-NTA beads and the expression levels were detected by an anti-FOXO1 antibody.
DISCUSSION

Mammalian FOXO factors play very important roles in development, glucose metabolism, cancer, aging, and energy homeostasis. Several signaling pathways regulate their activity, such as the insulin pathway, glucose, IGF1 and oxidative stress. In this study, we found that MDM2 is the E3 ubiquitin ligase for FOXO factors. MDM2 interacts with FOXO factors and promotes their ubiquitination and degradation. It also affects their biological function and downstream targets expression of FOXO.

1. MDM2 is the E3 ubiquitin ligase of FOXO.

FOXO proteins are known to be ubiquitinated and their level of expression is regulated by proteasome mediated degradation (Aoki et al., 2004; Plas and Thompson, 2003), but a general ubiquitin E3 ligase for FOXO proteins remains to be identified.

Skp2 was reported to inhibit FOXO1 in tumor suppression through ubiquitin-mediated degradation but such activity appears to be restricted to FOXO1 (Huang et al., 2005).

The present study identified MDM2 as a general ubiquitin E3 ligase for mammalian FOXO factors. We present multiple lines of evidences to support this conclusion. First, the manipulation of MDM2 expression by genetic deletion, knock down MDM2 with siRNA or overexpression of MDM2 using transient or stable transfection leads to change of the level of FOXO1 and FOXO3A proteins in opposite directions. Second, the down regulation of FOXO1 protein by MDM2 is relieved by treatment with a proteasome inhibitor, MG132, and the half-life of FOXO1 protein is decreased by ectopic
MDM2. Third, MDM2 binds to FOXO1 and FOXO3A and promotes their ubiquitination. The ubiquitination depends on both the ability of MDM2 to bind FOXO proteins as well as its E3 ligase activity. This is supported by the evidence that mutant MDM2 (∆150-230) that is missing in the FOXO binding region did not stimulate FOXO1 ubiquitination although it contains an intact E3 ring finger domain. Moreover, MDM2 mutants with either deletion {MDM2 (1-361)} or mutation {MDM2 E3-mt (457S)} at the C-terminus ring finger region lost their ability to stimulate FOXO ubiquitination even though they are still able to interact with FOXO1. Finally, in test tube reaction, recombinant MDM2 catalyzed the ubiquitination of FOXO1, supporting a direct substrate-enzyme relationship.

2. MDM2-promoted ubiquitination of FOXO depends on phosphorylation on PKB sites.

In response to stimulation by IL-3, insulin or PDGF (platelet derived growth factor), the activated PKB oncogene triggered proteasome-dependent degradation of its substrates including FOXO1 and FOXO3A (Aoki et al., 2004; Matsuzaki et al., 2003; Plas and Thompson, 2003). In our study, ubiquitination was found to depend on phosphorylation at sites that mediate the cytoplasmic localization of FOXO factors by PKB. FOXO1 (AAA), in which all three PKB sites are mutated to non-phosphorylatable alanine, did bind to MDM2 (Figure 19) but its level of ubiquitination was not affected by MDM2 (Figure 29). Similarly, ubiquitination of FOXO3A (AAA) was unaffected by MDM2 (Figure 30). These data suggest that MDM2 acts as a conditional E3 ligase for FOXO proteins, which is only functional after FOXO phosphorylation at AKT sites. Previous studies (Feng et al., 2004; Zhou et al., 2001) showed that phosphorylation of MDM2 by PKB inhibits its interaction with ARF, thereby increasing its stability and promoting its ability to bind and degrade p53. It remains to be determined whether the MDM2 phosphorylation active PKB regulates its ability to stimulate FOXO ubiquitination.
3. **MDM2-promoted ubiquitination of FOXO happens mainly in the cytoplasm.**

Protein ubiquitination and degradation occur in both the nucleus and cytoplasm (Yu et al., 2000). In our analysis, the nuclear MDM2 in which the nuclear export sequence is mutated contains an intact E3 region and is able to bind FOXO1 but unable to increase FOXO1 ubiquitination, on the other hand, the cytoplasmic MDM2, both the MDM2 NLS-mt and MDM2 (Δ89-150), stimulated ubiquitination, suggesting that it is the cytoplasmic MDM2 that functions as an E3 ligase for FOXO factors. In combination with the data that showed that nuclear FOXO1 (AAA) is not ubiquitinated by MDM2, it is reasonable to assume that FOXO ubiquitination occurs in the cytoplasm. However, such a conclusion might seem incompatible with the immunofluorescence staining data showing that the colocalization of MDM2 and FOXO occurred mainly in the nucleus. It should be pointed out that the colocalization studies were performed in cells grown in a medium containing low levels of serum, which minimizes PKB activity and inhibits FOXO cytoplasmic localization and degradation. Consistent with the degradation in the cytoplasm, the interaction between cytoplasmic MDM2 and FOXO1 was difficult to detect unless proteasome activity was inhibited by MG132. The fact that MDM2 NLS-mt that cannot enter the nucleus interacting with FOXO1 and stimulating its ubiquitination suggests that a nuclear interaction between MDM2 and FOXO proteins is not required for FOXO ubiquitination. Presently, the details of the nuclear interaction between MDM2 and FOXO proteins are unclear. It is possible that nuclear interaction between FOXO factors and MDM2 may exert an effect on FOXO factors that is distinct from degradation. In the case of p53 ubiquitination, it has been shown that monoubiquitination by MDM2 promotes the cytoplasmic localization of p53 whereas the polyubiquitination stimulates the degradation of p53 in nucleus (Li et al., 2003a).
4. **MDM2 affects both the transcriptional activity and ubiquitination of FOXO.**

FOXO factors are transcription factors. On one hand, FOXO1, FOXO3A and FOXO4 directly bind to the promoters of TRAIL, Bim, p27, MnSOD, GADD45, G6Pase, PEPCK, TAT and IGFBP-1 and of others. On the other hand, FOXO can also indirectly regulate other genes, such as cyclin D1. In our studies, a positive effect of MDM2 on the transcriptional activity of FOXO1 was detected in transient transfection reporter assays. It also promoted the ubiquitination and degradation of FOXO factors, leading to FOXO protein level decreases. It is not fully understood as to how MDM2 decreases FOXO proteins expression level. There are three possible mechanisms that might be responsible for MDM2’s positive effect on transcriptional activity of FOXO: 1) MDM2 promotes the transcriptional activity first, followed by its role in FOXO degradation because transcriptional activity data in H1299/V138 cells showed that FOXO activity was transiently induced between 5h and 48 h post p53 activation and subsequently FOXO activity decreased (Figure 47a); 2) FOXO4 was recently shown to be monoubiquitinated, which increases its transcriptional activity (van der Horst et al., 2006) and this raises the second possibility. MDM2 plays two roles in the regulation of FOXO factor. It may promote FOXO transcriptional activity through monoubiquitination but degrade FOXO protein through polyubiquitination; 3) About 20 proteins are reported to interact with MDM2, and p300 is reported to play a critical role in the MDM2-directed turnover of p53 (Grossman et al., 1998) and this raises the third possibility. Different cofactors are involved in the regulation of FOXO factors by MDM2 leading to different outcomes.

In our p53 study, Mdm2-mediated monoubiquitylation of p53 greatly promoted its mitochondrial translocation and thus its apoptosis in the mitochondria. Upon entrance in the mitochondria, p53 undergoes rapid deubiquitylation by mitochondrial HAUSP via a stress-induced mitochondrial p53-HAUSP complex (Marchenko et al., 2007). FOXO4
was also reported to become monoubiquitinated in response to increased cellular oxidative stress, resulting in its re-localization to the nucleus and an increase in its transcriptional activity. Deubiquitination of FOXO is catalyzed by the deubiquitinating enzyme HAUSP (herpesvirus-associated ubiquitin-specific protease), which interacts with and deubiquitinates FOXO in response to oxidative stress. We found that MDM2 suppresses the expression of FOXO1 target genes and protects cells from FOXO1-induced apoptosis. These observations are consistent with the effect of MDM2 on the translocation change and degradation of FOXO factors. Further study is required to understand the biological effect of MDM2 promoted transcriptional activity of FOXO factors and to establish whether FOXO factors have any mitochondrial function.

5. Mammalian FOXO factors interact with p53.

Mammalian FOXO factors and p53 are tumor suppressors and the regulators of aging that act similarly in many ways. They induce apoptosis (Modur et al., 2002) and cell cycle arrest (Medema et al., 2000; Nakamura et al., 2000; Schmidt et al., 2002) and regulate cellular responses to DNA damages and stress (Essers et al., 2004; Kajihara et al., 2006) through transcriptional induction of a similar set of target genes such as the p21 CDK inhibitor, Fas ligand and GADD45. In our study, we found that ectopically expressed p53 inhibited FOXO transcriptional activity in either DU145 cells (contain inactive p53) or LNCaP (contain wild type p53) but did not decrease FOXO1 induced cell death (Figure 44b). Two subsequent papers showed that p53 interacted with FOXO3A in the presence of stress and active FOXO3A could induce p53-dependent apoptosis and promote p53 cytoplasmic accumulation by increasing its association with the nuclear exporting machinery. All these evidence indicate that the regulation of transcriptional activity of p53 and FOXO factor is independent of the regulation of the proapoptotic activity of p53 and of FOXO factors.
p53 and MDM2 form a feedback loop, in which p53 induces MDM2 by activating MDM2 transcription, and MDM2 in turn negatively regulates p53 by binding and promoting p53 ubiquitination and degradation (Jin and Levine, 2001). This feedback loop keeps the p53 activity in check under normal conditions. Upon DNA damages, the interaction between MDM2 and p53 is suppressed, resulting in an increased p53 activity that triggers apoptosis. Our data suggest that MDM2 might act as a general coordinator to turn off multiple negative growth regulators when p53 senses that the DNA damage has been repaired (Figure 56). During our investigations, several studies documented a functional interaction between p53 and FOXO3A. In the presence of hydrogen peroxide, p53 and FOXO3A form a complex (Brunet et al., 2004). DNA damage promotes FOXO3A nuclear export through p53-dependent activation of serum and glucocorticoid activated kinases (You et al., 2004a). In endothelial cells (Miyauchi et al., 2004) and dermal fibroblasts (Kyoung Kim et al., 2005), inhibition of FOXO3A by PKB or siRNA promoted senescence-like growth arrest in a p53- and p21-dependent manner. Overall, p53 and FOXO factors appear to have a complex relationship, the outcome of which is likely to depend on cellular status and environment.

6. MDM2 regulates FOXO factors in a p53 independent way.

In addition to the negative regulation of p53 activity, MDM2 is overexpressed in tumor cells and functions as an oncogene to promote cancer cell growth independent of p53 (Daujat et al., 2001; Ganguli and Wasylyk, 2003). Activated FOXO3A was also reported to impair the transcriptional activity of p53, but enhanced its pro-apoptotic function in mitochondria (You et al., 2006b). The present studies suggest that suppression of FOXO factors may be one of the p53-independent mechanisms by which MDM2 promotes cancer cell survival and cell growth. Decreased FOXO3A and FOXO1
Figure 56 A working model shows the functional interaction among FOXO, p53 and MDM2.
activity from loss of PTEN led to a decrease in TRAIL expression and increased survival of prostatic tumor cells (Modur et al., 2002). Loss of p27 expression is associated with aggressive behavior in a variety of human epithelial tumors (Catzavelos et al., 1997; Macri and Loda, 1998). Stable expression of MDM2 in H1299 cells decreased the expression of FOXO3A together with TRAIL, p27 and MnSOD (Figure 35). Knockdown of MDM2 by siRNA in H1299 cells increased the level of FOXO3A together with TRAIL (Figure 36). These results suggest that MDM2 promotes tumorigenesis through down-regulation of FOXO target genes and that the targeted interruption of FOXO ubiquitination and degradation by MDM2 may represent an effective strategy for cancer prevention and therapy.

7. **Different domains of MDM2 play different roles in the regulation of p53 and FOXO factors (see Table 2).**

FOXO factor and p53 are the central regulators of cellular response to stress, genotoxic insult and DNA damage. MDM2 interacts with and promotes the degradation of p53. Our study shows that MDM2 also interacts with and promotes the ubiquitination and degradation of FOXO. But it became quite clear that different domains of MDM2 play different roles in the regulation of these two proteins. MDM2 (50-491), MDM2 (Δ50-89) and MDM2 (Δ 90-150) cannot bind with p53 and promotes its ubiquitination, but they do bind with FOXO and can promote the ubiquitination of FOXO factors. MDM2 (Δ 150-230) binds with p53 and promotes the ubiquitination of p53 but it does not interact with FOXO and promotes the ubiquitination of FOXO. The nucleo-cytoplasmic shuttling is critical for MDM2-promoted ubiquitination of p53 because even MDM2 (NES mutant) and MDM2 (NLS mutant) are capable of binding to p53, but cannot promote its
Table 2  Localization and function of different MDM2 mutants. ND- Not done yet; N-nucleus; C-cytoplasm.
ubiquination. MDM2 (Δ 222-437) is capable of binding to p53 and FOXO; as for ubiquitination, this mutant can only promote the ubiquitination of FOXO but not p53. One possible reason for this effect is the nucleolar localization of MDM2 (Δ 222-437). MDM2 (1-361) is a mutant which is deleted of the two highly conserved RING finger motifs in the C-terminus. MDM2 (457S) carries a mutation critical for E3 function of MDM. It prevents the polyubiquitination of p53, significantly lowers the efficiency of MDM2 interaction with MDMX and promotes MDMX polyubiquitination. Both of these mutants can bind to p53 and FOXO factors but cannot promote their ubiquitination. This indicates the essential role of the E3 region of MDM2 in the ubiquitination of both p53 and FOXO.

8. The sumoylation status of SIRT1 affects the stability and activity of FOXO.

Acetylation affects protein degradation via ubiquitination pathway (Ito et al., 2002; Jeong et al., 2007; Li et al., 2002). In β-cells, acetylation of FOXO1 decreased its degradation via the ubiquitin pathway (Kitamura et al., 2005). The current study showed that sumoylation of wild type SIRT1 increased its deacetylase activity, wild type SIRT1, but not SIRT1 (734R), decreased the acetylation level and transcriptional activity of FOXO1. This study suggests that sumoylation of SIRT1 affects its ability to regulate the transcriptional activity of FOXO1. The study also raised the possibility that SIRT1 sumoylation may regulate the stability of FOXO1.

9. Genistein increases FOXO expression level through down-regulation of MDM2.

Genistein (5,7,4′-trihydroxyisoflavone), a kind of isoflavone, is now considered to be the primary anticancer component of soybeans. Experimental data showed that in H1299 lung cancer cells, genistein increases the FOXO1 protein expression level in a dose dependent manner, overexpression of MDM2 abolishes the effect of genestein on FOXO1 (Figure 54). The data indicates that genistein increases FOXO1 protein
expression level and this effect is MDM2-dependent. Previous data showed that genistein down-regulates MDM2 expression at both the transcriptional and posttranslational levels, independently of p53, in both human cancer cell lines and primary cells. Up-regulation of the tumor suppressor p21\(^{\text{Waf1/CIP1}}\) by genistein can be regulated by both p53 and FOXO factors. Thus, the inhibition of MDM2 expression by genistein may be essential for its antitumor activities.

10. **ARF differentially affects E3 function of MDM2 toward different substrates.**

p19\(^{\text{ARF}}\) is the product of an alternative open reading frame of the mouse \(\text{INK4a-ARF}\) locus. It is a known fact that ARF is an inhibitor of the MDM2 E3 ligase function on p53 (Zhang and Xiong, 2001). In our study, also it was found that ARF stimulated FOXO1 ubiquitination as well (Figure 55). The data indicate that ARF interaction with MDM2 differentially affects its E3 function toward different substrates rather than inactivating its E3 function in general. This effect is plausible because ARF does not directly interact with the RING domain of MDM2, which may be involved in recruiting E2. Binding of ARF to the acidic domain of MDM2 may simply alter its ability to properly orient E2 for the transfer of ubiquitin to certain substrates. In the case of p53, ubiquitin conjugation is blocked. In the case of FOXO1, ubiquitination is stimulated. There are two possible explanations for this. The first one is that ARF may stabilize MDM2 interaction with FOXO, which may account for increased FOXO1 ubiquitination. The second one is that ARF may also qualitatively stimulate the E3 activity of MDM2 toward FOXO. Further experiments using the in vitro ubiquitination system will be required to address this matter.
SUMMARY AND PERSPECTIVES

Cancer is a major health problem in the USA and worldwide. One third of the people suffer from some form of cancer and 20% of all deaths are cancer related. In developed countries, cancer care represents about 10% of total health costs. Since the incidence of cancer increases with age and people live longer, some important questions need to be addressed: Why is cancer more prominent in older people and should cancer treatment be different for the various age groups. Our studies focus on the regulation of FOXO factors which are key elements of tumor initiation and of the mechanisms that regulate an organism's lifespan. They are potential candidates to serve as molecular linker between longevity and cancer. FOXO factors also regulate numerous cellular processes, such as stress resistance, the cell cycle, apoptosis, DNA repair/metabolism and tumorigenesis.

Ubiquitination controls the stability/degradation of FOXO factors, although the mechanism is not clearly defined. Our data allow us to draw the following conclusions: (1) MDM2 binds directly to FOXO1. Specifically, the binding between MDM2 and FOXO1 is through the central region of MDM2 and the N-terminal region of FOXO1. Interestingly, FOXO protein phosphorylated by AKT is not required for this binding; (2) MDM2 binding promotes the ubiquitin-dependent degradation of FOXO1 and FOXO3A. Knockdown of MDM2 by siRNA caused accumulation of both FOXO1 and FOXO3A. MDM2 mediated polyubiquitination of FOXO appears to occur in the cytoplasm and is an AKT phosphorylation dependent; (3) Recombinant MDM2 catalyzes the ubiquitination of
FOXO1 in test tube reactions, demonstrating a direct substrate-enzyme relationship; (4) MDM2 affects the expression of downstream FOXO targets and protects cells from FOXO1 induced cell death; (5) p53 transiently induced the transcription activity of FOXO3A, followed by degradation of FOXO3A protein through MDM2.

In addition to the above conclusions, we also made several preliminary but potentially important observations that need to be investigated in further studies.

1) In the transient transfection experiments, MDM2 increased the transcriptional activity of both wild type FOXO factors and FOXO (AAA), which is no longer phosphorylated by AKT anymore. The molecular mechanism underlying this effect needs further clarification and monoubiquitination could play a decisive role. Other important questions to be addressed are: whether monoubiquityination of FOXO factors is induced by MDM2 and is yes, whether monoubiquitination and polyubiquitination play different roles in FOXO function; whether monoubiquitination increase the transcriptional activity of FOXO without an effect on protein stability; whether MDM2 levels has a differential effect on the ubiquitination status of FOXO; and whether cofactors, such as p300 and SIRT1, have any effect on the ubiquitination status of FOXO1.

2) Genistein increases the expression of FOXO1 protein in a dose dependent manner in H1299 lung cancer cells. This effect is abolished in the H1299 cells that stably express MDM2. Our data suggested that the anti-tumor effect of genistein may be mediated, at least in part, through the down-regulation of MDM2, causing the up-regulation of FOXO factors (our present data) and p53 (earlier data of others). It remains to be seen whether this effect of genistein on FOXO1 can be extended to other FOXO factors and other cells lines and if so, whether it only affects FOXO protein stability or through other mechanisms. It is also important to determine how genistein may affect downstream targets of FOXO factors.
3) It is an established fact that PKB and insulin promote FOXO ubiquitination and degradation. It remains to be elucidated whether MDM2 is required for the PKB and insulin promoted degradation of FOXO factors and whether the growth factors control the levels of mono- vs. poly-ubiquitination.

4) SIRT1 acts as a “double-edged sword” promoting survival of aging cells, but also increasing the cancer risk. Several studies had shown that acetylation inhibits FOXO protein degradation. One key question is whether SIRT1 affects MDM2 promoted ubiquitination and degradation, and if so, whether sumoylation mutant of SIRT1 will affect stability of FOXO since the mutant is associated with a decrease in its deacetylase activity and with reduced ability to inhibit the transcriptional activity of FOXO1.

5) ARF is known to inhibit MDM2-induced p53 ubiquitination, but enhance MDM2-induced MDMX ubiquitination. In our study, ARF promoted FOXO ubiquitination. It is very important to define the molecular mechanism behind the differential effect of ARF on the MDM2-induced ubiquitination of p53 and FOXO factors.

In summary, my thesis work is the first to identify MDM2 as an E3 ubiquitin ligase for FOXO factors and suggests that the targeted inhibition of MDM2 E3 activity may be a more effective strategy for tumor suppression than the current strategy that targets solely the interaction with p53.
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