Cross-talk among HMGA1 and FoxO1 in control of nuclear insulin signaling

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As a mediator of insulin-regulated gene expression, the FoxO1 transcription factor represents a master regulator of liver glucose metabolism. We previously reported that the high-mobility group AT-hook 1 (HMGA1) protein, a molecular switch for the insulin receptor gene, functions also as a downstream target of the insulin receptor signaling pathway, representing a critical nuclear mediator of insulin function. Here, we investigated whether a functional relationship existed between FoxO1 and HMGA1, which might help explain insulin-mediated gene transcription in the liver. To this end, as a model study, we investigated the canonical FoxO1-HMGA1-responsive IGFBP1 gene, whose hepatic expression is regulated by insulin. By using a conventional GST-pull down assay combined with co-immunoprecipitation and Fluorescence Resonance Energy Transfer (FRET) analyses, we provide evidence of a physical interaction between FoxO1 and HMGA1. Further investigation with chromatin immunoprecipitation, confocal microscopy, and Fluorescence Recovery After Photobleaching (FRAP) technology indicated a functional significance of this interaction, in both basal and insulin-stimulated states, providing evidence that, by modulating FoxO1 transactivation, HMGA1 is essential for FoxO1-induced IGFBP1 gene expression, and thereby a critical modulator of insulin-mediated FoxO1 regulation in the liver. Collectively, our findings highlight a novel FoxO1/HMGA1-mediated mechanism by which insulin may regulate gene expression and metabolism.

The regulation of glucose metabolism and homeostasis is a central component of living systems. In mammals, this function is performed through distinct but interrelated cell signaling pathways that mediate the transduction of hormonal and nutrient stimuli to the nucleus, resulting in modifications of nuclear regulatory proteins (transcription factors), which bind to specific sites on the DNA and cause the activation or repression of genes and gene networks involved in these metabolic processes. Among the nuclear factors that act downstream of these pathways, the forkhead box protein O1 (FoxO1) is an important nutrient-sensing transcription factor that modulates the expression and activity of insulin-sensitive genes involved in gluconeogenesis, glycogenolysis and energy homeostasis. Central to the functional regulation of FoxO1 is its nucleocytoplasmic shuttling following FoxO1 protein phosphorylation by the insulin-dependent phosphatidylinositol 3-Kinase/Akt (PI3K/Akt) signaling pathway, a process that is further enhanced by FoxO1 acetylation. Thus, in fasting conditions when insulin is low and the PI3K/Akt pathway is abrogated, FoxO1 binds the gluconeogenic genes, and activates their expression, contributing to the maintenance of fasting euglycemia. Vice versa, in fed conditions, phosphorylation of FoxO1 by insulin, leading to the detachment of FoxO1 from DNA and its shuttling into the cytosol, represses gluconeogenesis, thereby contributing to the maintenance of postprandial glucose homeostasis.

The high-mobility group A1 (HMGA1) protein is an architectural factor that binds to adenine-thymine (A-T) rich regions of DNA. By itself, HMGA1 has no intrinsic transcriptional activity; rather, it can transactivate promoters by facilitating the assembly and stability of higher-order transcriptional complexes – so-called enhosomes – that drive gene transcription in response to extracellular and intracellular signals. Such signals may affect HMGA1 function by inducing changes in post-translational protein modifications that markedly influence...
FoxO1 is an important mediator of insulin action at these levels, and cytoplasmic retention of FoxO1 via protein-protein interactions, that mediate transcriptional activation. As previously reported, both mechanisms: by post-translational modifications, which drive subcellular localization of FoxO1, and by transcriptional repression of gluconeogenic genes, is mediated by HMGA1. Insulin exerts this transcriptional repression by triggering HMGA1 phosphorylation at three serine residues, via a PI3K/Akt pathway, inducing transcriptional repression of gluconeogenic genes. On the other hand, a relationship between HMGA1 and the insulin receptor signaling system has been demonstrated before, showing that HMGA1 is a key regulator of the expression of the insulin receptor. Consistently with these observations, HMGA1 gene defects produce insulin resistance and type 2 diabetes in humans and mice, whereas protection against insulin resistance has been reported in transgenic mice overexpressing Hmga1.

Increasing evidences demonstrated that FoxO1 executes its function on gene promoters by interacting with other nuclear proteins with which it assembles different transcriptional complexes. However, the mechanisms by which these processes regulate FoxO1 activity are still largely unknown. On the base of the above considerations, here we explored the hypothesis that the insulin-mediated FoxO1-regulated metabolic pathway in the liver could be modulated by direct interactions of HMGA1 with FoxO1.

Results

Physical interaction between HMGA1 and FoxO1. We previously showed that phosphorylation of HMGA1 represents a critical event in mediating the insulin's effect on gluconeogenic genes. As previously reported, both mechanisms: by post-translational modifications, which drive subcellular localization of FoxO1, and by protein-protein interactions, that mediate transcriptional activation. As previously reported, both mechanisms are triggered by a wide variety of extracellular stimuli, which include oxidative stress, growth factors, and other nuclear proteins with which it assembles different transcriptional complexes. However, the mechanisms by which these processes regulate FoxO1 activity are still largely unknown. On the base of the above considerations, here we explored the hypothesis that the insulin-mediated FoxO1-regulated metabolic pathway in the liver could be modulated by direct interactions of HMGA1 with FoxO1.

Subcellular colocalization of HMGA1 and FoxO1.

Thus, these results clearly indicate that HMGA1 and FoxO1 physically interact with each other in vitro, in cell free extracts, as well as in the context of intact cells and liver tissue samples.

![Figure 1](https://www.nature.com/scientificreports/)
nutrients. To substantiate the interaction between HMGA1 and FoxO1, and to address their subcellular localization in living cells, we performed FRET studies that finely allow the detection of associated proteins in living cells in real time. To this end, the coding sequences of both HMGA1 (HMGA1a isoform) and FoxO1 (FoxO1a isoform) were tagged with the yellow fluorescent protein (YFP) and the cyan fluorescent protein (CFP), respectively, and expressed in HEK-293 cells that were deprived of serum (starved) to prevent nuclear/cytoplasmic shuttling of the FoxO1 protein. As determined by Western blot analysis and reporter gene assay, both fusion proteins were expressed and functionally active following transfection of expression plasmids into cells (Fig. 2a). As shown in Fig. 2b, YFP-HMGA1 and CFP-FoxO1 exhibited a robust FRET signal in the nucleus of serum starved HEK-293 cells, with an almost equivalent distribution between the heterochromatin chromatin domains, found near the nuclear envelope, and the more transcriptionally active central nuclear position, which supports the physical interaction between the two proteins at this level. HEK-293 cells were ideally suited for FRET experiments since
they do not express appreciable levels of endogenous HMGA1 and FoxO1, a condition that improves the analysis of transfected fluorescent proteins.

**Functional significance of HMGA1-FoxO1 protein-protein interaction.** The functional significance of the interaction between HMGA1 and FoxO1 in vivo was then investigated at the level of the endogenous IGFBP1 genomic locus, which is recognized as a downstream target of the insulin receptor signaling pathway, and whose transcriptional regulation by either FoxO1 or HMGA1 has also been reported. Using the IGFBP1 gene promoter as a target of insulin action, we performed ChIP assays in HEK293 cells, along with quantitative qRT-PCR of ChIP-ed samples. As shown in Fig. 3a, FoxO1 occupancy at the IGFBP1 chromatin target site was downregulated by insulin and was significantly reduced in cells pretreated with distamycin A, a DNA-binding agent which selectively blocks DNA binding by HMGA1. In line with our previous observations indicating that functional integrity of HMGA1 is required for normal insulin action, insulin failed to downregulate FoxO1-DNA binding in the presence of distamycin A (Fig. 3a). DNA occupancy by FoxO1 at these sites closely paralleled the changes in IGFBP1 protein production in HepG2 cells under the same experimental conditions as those used for ChIP (Fig. 3b), thus suggesting that binding of HMGA1 to DNA is required for FoxO1-DNA interaction and transcriptional activity and may constitute a prerequisite for the functional regulation of FoxO1 by insulin.

To clarify whether HMGA1/FoxO1 interaction had a functional implication in the transcriptional activity of IGFBP1 promoter, HEK-293 cells, which barely express HMGA1, were transfected transiently with an IGFBP1 luciferase reporter construct (IGFBP1-Luc), in the presence of both HMGA1 and FoxO1 expression vectors. As shown in Fig. 4a, simultaneous overexpression of HMGA1 and FoxO1 in HEK-293 cells led to a significant increment in IGFBP1-Luc activity that exceeded that seen with either factor alone, resulting in synergistic activation of transcription. Higher amounts of HMGA1 failed to increase further IGFBP1-Luc activity probably due to the property of HMGA1 to bind non-specifically to the DNA when present at high concentrations. As previously reported, insulin elicited no effect on this mutant protein, which remained phosphorylatable alanines. As previously reported, insulin elicited no effect on this mutant protein, which remained phosphorylatable alanines. As previously reported, insulin elicited no effect on this mutant protein, which remained phosphorylatable alanines. As previously reported, insulin elicited no effect on this mutant protein, which remained phosphorylatable alanines.
Figure 4. HMGA1/FoxO1 interaction and IGFBP1 gene transcription. (a) HEK-293 cells, barely expressing endogenous HMGA1, were cotransfected with IGFBP1-Luc reporter vector, plus FoxO1 expression plasmid (0.1 μg), either in the absence or presence of increasing amounts (0, 0.25, 1 μg) of effector vectors for HMGA1 wild-type (HMGA1, gray bars) or mutant HMGA1 (HMGA1m, slashed bars) (0.25 μg). At 48 h after transfection, cells were incubated in the absence (−) or presence (+) of insulin and cell lysates were prepared 4 h later. Cell lysates were divided into two aliquots; one was used for Luc activity, and the other for WB analysis as a control of HMGA1 protein expression. Values of Luc activity in each condition are expressed as factors by which reporter activity increased or decreased as compared to the level of Luc activity obtained in transfections with IGFBP1-Luc reporter vector alone (black column), which is assigned an arbitrary value of 1. White bar, pcDNA3 vector without an insert. Data represent the means ± s.e.m. for three separate experiments. Representative WBs of endogenous and overexpressed HMGA1a and HMGA1m proteins are shown. *P < 0.05 vs control (black bar); **P < 0.05 vs relative insulin-untreated cells. (b) Human IGFBP1-Luc reporter vector (2 μg) was cotransfected with 0.1 μg of FoxO1 effector plasmid into HepG2 cells pretreated with anti-HMGA1 siRNA or a nontargeting control siRNA. At 48 h after transfection, cells were incubated in the absence (−) or presence (+) of insulin, cell lysates were prepared 4 h later, and Luc activity was measured. Data represent the means ± s.e.m. for three separate experiments; Luc activity in each condition is expressed as a percentage of the reporter activity obtained in transfections with IGFBP1-Luc reporter vector, in the presence of FoxO1 effector vector alone. White bar, mock (no DNA); black bar, pcDNA3 basic vector (without an insert). Representative WBs of HMGA1 in each condition are shown. β-actin, control of protein loading. Cropped blots are shown in the figures. Full-length WBs are presented in Supplementary Fig. S1.

Dynamic interaction of HMGA1 with FoxO1. We next sought to investigate the dynamic interaction of HMGA1 and FoxO1 proteins in living cells. To this end, FRAP experiments were carried out in cells transfected with the CFP-tagged FoxO1 construct. By bleaching CFP-FoxO1 fluorescence in a defined nuclear region, we determined the time taken for fluorescence to recover by time-lapse confocal microscopy (Fig. 5a). Taking into account that the fluorescence recovery is dependent on the movement of unbleached CFP-FoxO1 molecules from the surrounding environment into the bleached region, it was then possible to measure the mobility of FoxO1 nuclear protein in CFP-FoxO1-expressing cells. In serum-starved HEK-293 cells, the fluorescence recovery of nuclear CFP-FoxO1 was incomplete (40% recovery), with a recovery time (t1/2) of 1.26 s, indicating that a significant fraction (~60%) of the CFP-FoxO1 fluorescent protein was bound to an immobile element within heterochromatic clusters (Fig. 5b). This observation fitted well with the notion that, in basal (starved) conditions, FoxO1 preferentially bound to DNA. Overexpression of either HMGA1 or HMGA1m in HEK-293 cells did not affect neither the fraction of immobile CFP-FoxO1 molecules (P = 0.28 and P = 0.71, respectively), nor the time of recovery for CFP-FoxO1 (P = 0.32 and P = 0.38, respectively) (Fig. 5b). After insulin treatment, the fluorescence recovery of CFP-FoxO1 was up to ~60%, with a shorter recovery time (t1/2 = 0.91 s), as compared to unstimulated cells (P < 0.001), thus indicative of an increased nuclear mobility of the CFP-tagged FoxO1 protein (Fig. 5b), which is consistent with the insulin-induced nucleocytoplasmic shuttling of FoxO1. Overexpression of HMGA1 in HEK-293 cells under insulin treatment reduced the immobile fraction of CFP-FoxO1 to 46.7%, suggesting an increasing number of CFP-FoxO1 molecules being tethered within heterochromatic clusters, whereas similar kinetics of dissociation of CFP-FoxO1 from endogenous and over-expressed HMGA1 were observed (FRAP recovery time: t1/2 = 1.65 s) (Fig. 5b). Instead, in cells overexpressing the mutant form of HMGA1 (HMGA1m), the recovery time of CFP-FoxO1 was 3.37 s, significantly higher (P < 0.01) with respect to control cells expressing the wild-type HMGA1 protein, thus indicating a reduced mobility of FoxO1, and an impaired nucleocytoplasmic shuttling of phosphorylated FoxO1 (Fig. 5b). Increased nuclear retention of phosphorylated FoxO1 was confirmed in time course experiments of FoxO1 protein phosphorylation in cytosolic and nuclear fractions of HEK-293 cells overexpressing either the wild-type or the mutant HMGA1m protein, which underwent insulin treatment (Fig. 5c). These data were further substantiated by ChIP assays coupled with qRT-PCR of ChIP-ed samples, showing that binding of FoxO1 to the endogenous IGFBP1 chromosomal locus was increased in HEK-293...
Figure 5. Insulin-induced HMGA1/FoxO1 phosphorylation and their kinetics. (a) Time lapse imaging of the intranuclear distribution of CFP-FoxO1 in HEK-293 cells, either untransfected (control), or transfected with HMGA1 or HMGA1m, after treatment (0 and 20 min) with insulin. Pictures are optical sections made with a confocal laser scanning microscope. Bars correspond to 2 µm. Representative images of CFP-FoxO1 in HEK-293 cells relative to the FRAP experiment reported in (b). (b) FRAP analysis of FoxO1 in HEK-293 cells overexpressing HMGA1 wild-type or mutant HMGA1m. FRAP curves of CFP-FoxO1 in HEK-293 cells, either untreated (left) or treated with 10 nM insulin for 20 min (right). FRAP curves were calculated as described in Methods. (c) Time-course of the effect of insulin on FoxO1 phosphorylation in HEK-293 cells overexpressing HMGA1 (left) or HMGA1m (right). Representative WBs of HMGA1 and nuclear and cytosolic phosphorylated pFoxO1 are shown in both conditions. A comparison of the nuclear phosphorylated form of FoxO1 in cells overexpressing either HMGA1 (black bars) or HMGA1m (gray bars), under insulin treatment, is shown in bar graphs. Data are shown as the means ± s.e.m. of three separate experiments. Cropped blots are shown in the
cells overexpressing HMGAI, in which the nuclear levels of pFoxO1 were higher with respect to cells overexpressing the wild-type form of HMGAI (Fig. 5d). In line with this, as shown in Fig. 5e, IGFBP1 mRNA and protein levels were lower in insulin-treated HEK-293 cells overexpressing HMGAI wild-type and higher in cells overexpressing the mutant HMGAIm protein, with these latter cells turning refractory to insulin with regard to the inhibition of IGFBP1.

Thus, taken together, these findings well support the functional relevance of HMGAI-FoxO1 interaction and provide evidence for the involvement of HMGAI in insulin-mediated nucleocytoplasmic shuttling of FoxO1 protein.

**FoxO1 and IGFBP1 expression in primary hepatocytes from normal and Hmgal-deficient mice.**

To further explore the functional significance of HMGAI-FoxO1 interaction in nuclear insulin signaling, we then performed experiments in primary cultured hepatocytes from both wild-type and Hmgal−/− mice. In these experiments, mRNA expression of FoxO1 and Igfbp1 was measured in primary cultured cells that were pretreated with the casein kinase (CK) 2 inhibitor 4,5,6,7-tetabromo-1H-benimidazole (TBB), which selectively blocks insulin-induced HMGAI phosphorylation, and the subsequent detachment of HMGAI from DNA23, without affecting FoxO1, whose phosphorylation is, instead, dependent on CK1 and several other protein kinases that alter subcellular location of FoxO1, DNA binding and transcriptional activity42–45. Consistent with previous observations from our group34,40,46, mRNA expression of FoxO1 and Igfbp1 was lower in primary cultured Hmgal−/− hepatocytes than in wild-type hepatocytes (Fig. 6). Following insulin treatment, the expression of mRNA for both FoxO1 and Igfbp1 decreased to 40–50% of control in cells from wild-type mice, whereas no changes in mRNA were observed in insulin-treated cells from mutant animals. Moreover, pretreatment of wild-type hepatocytes with TBB substantially attenuated the cellular response to insulin, with a less pronounced reduction in mRNA levels (Fig. 6), thereby indicating that the insulin's effect on these two genes is mediated by HMGAI, and that functional cooperation between HMGAI and FoxO1 may indeed constitute an essential step in nuclear insulin action.

**FoxO1 in vivo, in normal and Hmgal mutant mice.** As a further step toward understanding the relationship between HMGAI and FoxO1, we extended the above in vitro studies to studies in vivo, in wild-type (Hmgal+/−) and Hmgal−/− mice. As shown in Fig. 7, mRNA and protein abundances for FoxO1 were reduced in liver from 12 h-fasted Hmgal−/− mice, compared with wild-type animals. After refeeding, when the insulin receptor signaling is reactivated, both FoxO1 mRNA and protein were significantly reduced in liver from wild-type animals, while no difference on these parameters was observed in Hmgal−/− mice (Fig. 7), thereby confirming not only that HMGAI is necessary for FoxO1 expression in vivo, in whole animals, but also that insulin-triggered FoxO1 downregulation is mediated by HMGAI. Similar results were obtained with mRNA and protein expression of Igfbp1 in liver from wild-type and Hmgal−/− mice under the same experimental conditions than those used for FoxO1 expression (Fig. 7), thus supporting the role of HMGAI in the transcriptional regulation of IGFBP1 through the insulin/FoxO1 pathway.

The above results were confirmed by studies examining FoxO1 DNA-binding activity in vivo, in both wild-type and Hmgal−/− mice. As shown by ChIP in whole liver tissue, and subsequent qRT-PCR of ChIP-ed samples, binding of the Igfbp1 gene promoter by endogenous FoxO1 was reduced in Hmgal−/− mice compared to Hmgal+/− animals (Fig. 8). After insulin injection in living mice, binding of FoxO1 to DNA was significantly decreased in wild-type mice, while no differences were observed in Hmgal−/− mice (Fig. 8). The same results were replicated in mice after meal ingestion, when endogenous insulin levels are expected to increase. As shown in Fig. 8, binding of FoxO1 to the Igfbp1 locus was high in wild-type mice under fasting conditions when nutrients are limited, serum insulin is decreased and binding of HMGAI to DNA is preferentially increased. Conversely, FoxO1-DNA interaction promptly decreased in wild-type mice after refeeding, when serum insulin increases, insulin signaling is reactivated and both FoxO1 and HMGAI become phosphorylated (Fig. 8). In fasted Hmgal−/− mice, binding of FoxO1 to the Igfbp1 promoter was lower, compared to that in wild-type mice, but in contrast to normal animals, no change in FoxO1-DNA interaction was detected upon refeeding of mutant animals (Fig. 8), thus confirming that HMGAI is indeed required for the functional regulation of FoxO1 by insulin in vivo. Consistent with the above observations, fasting glycemia was significantly lower in Hmgal−/− null mice, compared to wild-type animals (Fig. 9), confirming the results obtained in previous studies using a large number of both wild-type and Hmgal−/− mice.40,46,47.
Discussion

As a member of the forkhead family of transcription factors, FoxO1 regulates the transcription of a wide variety of genes involved in fundamental biological processes, such as cell growth and differentiation, DNA repair and apoptosis, inflammation and immune response.1,2 Also, FoxO1 is a critical regulator of insulin signal transduction and energy metabolism.3,4 In this last context, cytoplasm retention of FoxO1 via insulin-induced phosphorylation is considered to be a mechanism of insulin-mediated gene expression and regulation.1 Dysregulation of FoxO1 at this level has been linked to metabolic abnormalities, including insulin resistance and type 2 diabetes.5 Therefore, the observation that an interplay among HMGA1 and FoxO1 can be a component of the insulin/FoxO1 signaling pathway constitutes a novel point of the present study, which may help in understanding the molecular basis of certain disorders where insulin action becomes compromised (e.g. obesity, type 2 diabetes and other insulin-resistant conditions).

For the first time in the present work, we demonstrate that HMGA1 physically and functionally interacts with FoxO1, thereby regulating both FoxO1-DNA binding and insulin-mediated FoxO1 gene transcription. Although a direct interaction between FoxO1 and HMGA1 has been observed in our study, in the absence of DNA, we show that binding of HMGA1 to the insulin target gene IGFBP1 is necessary for FoxO1-DNA binding and transcriptional activity, and this may constitute an important requisite for the functional regulation of FoxO1 by insulin. In fact, when the ability of HMGA1 to bind DNA was prevented by distamycin A in vitro, in cultured cells, binding of FoxO1 to target DNA was markedly reduced, endogenous FoxO1-dependent gene expression was decreased, and insulin failed to downregulate FoxO1-DNA binding. Conversely, FoxO1-DNA binding and transcriptional activity were increased in cells overexpressing the mutant HMGA1m lacking the three serine phosphorylation sites responsive to insulin, in which, instead, the phosphorylation capability of FoxO1 by the PI3K/Akt pathway was not modified. Consistent with our results supporting a role for HMGA1 in nuclear FoxO1 mobility, nucleocytoplasmic shuttling of insulin-activated FoxO1 was impaired in cells overexpressing the mutant HMGA1m, whereas the nuclear trapping of FoxO1 was increased, thus providing compelling evidence that HMGA1 is indeed an important factor in modulating FoxO1 activity in the context of insulin receptor signaling and that the dynamic interaction between HMGA1 and FoxO1 plays a critical role in the control of insulin-mediated gene expression. In this regard, we previously reported that the counter-regulatory hormone glucagon, which acts in opposition to insulin to maintain fasting euglycemia, upregulated HMGA1 expression via the CAMP pathway, both in vitro and in vivo in whole mice.47,48 Consistent with previous observations, and with the observation that HMGA1 activates FoxO1 gene expression,46,47, upregulation of FoxO1 via the glucagon-cAMP-PKA signaling has been reported in liver of fasting mice to maintain fasting euglycemia.49 Based on our findings here, it is likely that upregulation of HMGA1 during fasting (when glucagon peaks) may contribute to the maintenance of fasting euglycemia through two distinct but converging mechanisms: by increasing FoxO1 expression in response to glucagon, and by directly and dynamically interacting with FoxO1, thus playing an important role in fine-tuning the activation of FoxO1’s transcriptional activity. Exactly the opposite after meal, when insulin peaks in response to high glucose availability and glucagon is suppressed. In this metabolic setting, functional inactivation of HMGA1 following insulin-induced HMGA1 protein phosphorylation, by causing the detachment of FoxO1 from DNA and its inactivation by nuclear exclusion, represses hepatic gluconeogenesis, thereby contributing to the maintenance of postprandial glucose homeostasis.

The functional affinity between HMGA1 and FoxO1 is also underlined by the fact that other than phosphorylation, both proteins are also post-translationally regulated by the same histone acetyltransferase CBP (CREB-binding protein), which was found to catalyze the acetylation of both HMGA1 and FoxO1, with subsequent inactivation of transcriptional activity of both nuclear factors.50,51. On the other hand, distinct roles of HMGA1 and FoxO1 in the regulation of pancreatic beta-cell function and insulin production have been reported...
by us and others. However, it cannot be excluded the possibility that functional interaction of HMGA1 with FoxO1 may be cell specifically regulated by additional factors, so that cooperation between HMGA1 and FoxO1 in the transcriptional regulation of FoxO1-target genes is essential in some cell types, but not in others.

The possibility for a functional relationship between HMGA1 and FoxO1 is further supported by some metabolic similarities between Hmga1-deficient mice and liver-specific FoxO1-deficient mice, including insulin hypersensitivity of peripheral tissues following insulin tolerance test, and lower fasting plasma glucose levels as compared with wild-type animals. Similarities in body weight and body composition profiles are also found in both knockout mouse models, in which no changes were observed in these parameters among normal and mutant mice. Also, similarly to what was observed before, in mice lacking liver FoxO1, a trend in increasing serum cholesterol levels was seen in Hmga1-knockout mice compared with controls, with a trend in reduction of triglycerides (unpublished data). Concerning this last point, it has to be admitted that conflicting results have been reported in liver-specific FoxO1-deficient mice, in which plasma triglycerides were either unaffected or increased.

In toto, these findings establish HMGA1 as a critical element in the functional activity of FoxO1 transcription factor. Also, the data indicate that cross-talking between HMGA1 and FoxO1 is critical in relaying insulin signals down to the DNA, thereby ensuring insulin's transcriptional regulation of glucose metabolism and homeostasis. Understanding this cross-talk is interesting from both biological and mechanistic standpoints and might be useful in understanding the molecular basis of disease states that arise from alterations in nuclear insulin signaling.
Methods

Ethics statement. The study was approved by the local ethics committee, Regione Calabria Comitato Etico Sezione Area Centro (protocol registry n. 116 of May 14, 2015), and the methods were performed in accordance with approved guidelines. All animal work was performed using approved animal protocols, according to relevant institutional guidelines for the care of laboratory animals (directive 86/609/ECC, European Community Council).

Cell cultures and nuclear extracts. Human embryonic kidney 293 (HEK-293) cells and HepG2 human hepatoma cells were cultured in DMEM and RPMI 1640 medium, respectively, supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified 5% CO₂ atmosphere at 37 °C. Cytoplasmic and nuclear protein extracts were prepared as previously24,65 and final protein concentration in the extracts was determined by the modified Bradford method (Bio-Rad Laboratories) 66.

Glutathione S-transferase (GST) pull-down assay and co-immunoprecipitation. ³⁵S-labeled FoxO1 protein was synthesized by using the TNT-T7 quick-coupled in vitro transcription/translation system (Promega). GST tagged human HMGA1 was obtained using the pcDNA1-GST/HMGA1 expression vector, a kind gift from D. Thanos (Institute of Molecular Biology, Genetics and Biotechnology, Athens, Greece)24. Covalent coupling of antibody to protein A-Sepharose (GE Healthcare) was performed as previously described 67. Antibody-coupled protein A beads were washed twice in phosphate-buffered saline and used in immunoprecipitation studies. Briefly, aliquots of HepG2 cell nuclear extract or pure HMGA1 were incubated for 3 h with rotation at 4 °C with 10 µl of antibody coupled protein A beads. Beads were recovered by gentle centrifugation and washed three times with 500 µl of NETN wash buffer [50 mM Tris-HCl (pH 8.0), 0.1% NP-40, 150 mM NaCl, 1 mM EDTA] for 5 min. Protein was removed from the beads by boiling in sample buffer for 5 min and analyzed by SDS-PAGE and immunoblotting 67. Antibodies used for these studies were as follows: anti-HMGA124 and anti-FoxO1 (sc-11350) (Santa Cruz Biotechnology).

Plasmid construction and transfections, Fluorescence Resonance Energy Transfer (FRET), Fluorescence Recovery After Photobleaching (FRAP) analysis and confocal microscopy. Recombinant Luc reporter construct containing the human IGFBP1 gene promoter has been described previously23. The construct was transiently transfected into HEK-293 or HepG2 cells, using the LipofectAMINE 2000 reagent (Invitrogen Life Technology Corporation), in the presence or absence of effector vector for HMGA1 (HMGA1a isoform protein)23 and/or FoxO168, or HMGA1am 23, and Luc activity was assayed 48 h later in a luminometer (Turner Biosystems Inc.), using the dual-luciferase reporter assay system (Promega). siRNA targeted to human HMGA1 (sc-37115) was purchased from Santa Cruz Biotechnology, whereas nonspecific siRNA controls with a similar GC content was obtained from Dharmacon (GE Dharmacon). 100–200 pmol siRNA duplex was transfected into cells at 40–50% confluency. After knockdown for 72 h, cells were trypsinized, pooled, and resuspended for a second transfection using the same targeting siRNA. After an additional 72 h, cells were prepared for analysis. Renilla control vector served as an internal control of transfection efficiency, together with measurements of protein expression levels.

To produce YFP-HMGA1a and CFP-FoxO1 expression plasmids, the human HMGA1a ORF (NCBI Ref. Seq. NM_145899.2) was cloned into BamHI/XbaI sites of pYFP (Clontech) and the human FoxO1 ORF (NCBI Ref. Seq. NM_002015.3) was cloned into BamHI/XbaI sites of pCFP (Clontech). Confocal imaging was performed with Leica SP2 inverted confocal microscope (Leica Microsystems), using 63 × Apo PLA oil immersion objective.
objective (NA 1.4) and 476 nm laser line. A 1 mm² squared ROI within nucleus in heterochromatic region was set up for bleaching, 4 mm² ROI for bleaching correction, and additionally 4 mm² control ROI outside of cell area was acquired for background subtraction. The laser intensity was set up at 15%. Ten images were taken before the bleach pulse (15 laser iterations with bleaching intensity output 100%) and 60 images were taken after the bleaching with image acquisition each 0.3 s with 15% laser transmission. All FRAP experiments were performed in temperature-controlled CO₂ supplied chamber. All FRAP plots were generated from background subtracted images using the public domain software ImageJ as previously described. Shortly, the signal I was calculated in the area of interest (AOI) and normalized to the change in the total fluorescence due to the bleach pulse and the imaging: I = (To/Jo) * It/Tt, where To and Tt are the total cellular fluorescence in the pre-bleach and post-bleach images, respectively, whereas Jo is the average intensity in the AOI in the pre-bleach image.

**Chromatin immunoprecipitation (ChIP).** ChIP assay was performed in cultured HepG2 and HEK-293 cells, either untreated or pretreated with siRNA targeting HMG1A, as described previously. For in vivo ChIP, at the end of the indicated treatments, mice were killed by cervical dislocation, the liver was rapidly removed, prewarmed with PBS, and treated with 0.2% collagenase for 15 min. The liver was then diced, forced through a 60 μm stainless steel sieve, the hepatocytes were collected directly into DMEM containing 1% formaldehyde, and the formaldehyde-fixed DNA-protein complexes were immunoprecipitated with the anti-FoxO1 antibody sc-11350 from Santa Cruz Biotechnology. Sequence-specific primers for the Igfbp1 gene promoter used for PCR amplification of ChIP-ed DNA (30 cycles), using PCR ready-to-go beads (Amersham Pharmacia Biotech): human Igfbp1 (NT_007819) for 5′-CAGAAAGAGGAACATCCG-3′, rev 5′-TACACCAGGACGCGAGCA-3′; mouse Igfbp1 (NT_039515) for 59-CCTGGGGAGGAGAAACAGCTC-39, rev 59-GCAGTTCTAATGTGGTGG-39. PCR products were electrophoretically resolved on 1.5% agarose gel and visualized by ethidium bromide staining.

**Immunoprecipitation and Western blot.** A polyclonal-specific antibody against HMG1A was used to analyze HMG1A protein expression in HEK-293 and HepG2 cells, and in liver nuclear extracts from normal and Hmga1 mutant mice. An anti-FoxO1 antibody (Santa Cruz Biotechnology) was employed to measure total FoxO1 expression by insulin. The role of winged helix/forkhead proteins. Evidence for insulin response unit-dependent and –independent effects of insulin on promoter activity. Male Hmga1−/− mice and wild-type littermates aged 6–9 months were studied. The generation of these animals has been described previously. We have thoroughly investigated male mice to exclude the known effects linked to estrogen cyclicity, thereby limiting variability. FoxO1 and Igfbp1 mRNA and protein levels were analyzed in liver from age and weight matched 12-h fasted mice and after 4 h refeeding. ChIP of the Igfbp1 gene was performed in liver from 12 h-fasted animals following intraperitoneal injection of insulin (1U/kg bw) or saline, and after fasting and refeeding conditions. Cryopreserved primary hepatocytes from Hmga1−/− and wild-type mice were cultured as previously described.

**Statistical analysis.** The non-parametric Mann-Whitney test was used for comparisons of continuous variables. A significance level of 0.05 was set for a type I error in all analyses. All bar graph data shown represent mean ± standard error of the mean (s.e.m.). All data were analyzed with SPSS 20.0 software (SPSS Inc.).

**Data availability.** The datasets generated during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**

E.C. and A.B. conceived the study, designed the experiments, and analyzed the data; E.C. performed statistical analysis and prepared the first draft of the manuscript; B.A. performed transfection studies and ChiP; D.M.C. performed western blot analyses, pull-down and co-immunoprecipitation studies; C.P. performed FRAP and FRET experiments; V.M.M. and D.B. were involved in the data analysis and biological interpretation; M.A. provided the effector vector for FoxO1; D.P.F. was involved in data analysis, interpretation and critical reading of the manuscript; A.B., the corresponding author, provided financial support, supervised the project and wrote the final version of the paper.

**Additional Information**

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