The Wnt/β-Catenin→Pitx2 Pathway Controls the Turnover of Pitx2 and Other Unstable mRNAs

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Summary

The Wnt/β-catenin pathway rapidly induces the transcription of the cell-type-restricted transcription factor Pitx2 that is required for effective cell-specific proliferation activating growth-regulating genes. Here we report that Pitx2 mRNA displays a rapid turnover rate and that activation of the Wnt/β-catenin pathway stabilizes Pitx2 mRNA as well as other unstable mRNAs, including c-Jun, Cyclin D1, and Cyclin D2, encoded by critical transcriptional target genes of the same pathway. Our data indicate that Pitx2 mRNA stabilization is due to a reduced interaction of Pitx2 3′UTR with the destabilizing AU-rich element (ARE) binding proteins (BPs) KSRP and TTP as well as to an increased interaction with a stabilizing ARE-BP, HuR. Pitx2 itself is a mediator of Wnt/β-catenin-induced mRNA stabilization. Our previous and present data support the hypothesis that a single pathway can coordinately regulate sequential transcriptional and posttranscriptional events leading to an integrated functional gene regulatory network.

Introduction

Many tissue-restricted transcription factors mediate crucial steps during development functioning as the distal targets of different classes of regulatory signaling pathways (Dasen and Rosenfeld, 2001). One of these, the Wnt signaling cascade, controls organogenesis by inducing a wide range of responses from cell proliferation to cell fate determination and terminal differentiation (Cadigan and Nusse, 1997). Extracellular Wnt signals activate the cytoplasmic protein Dishevelled (Dvl) that, in turn, inhibits the constitutive proteasomal destruction of β-catenin (Boutros and Mlodzik, 1999). As a result, β-catenin accumulates in the nucleus, associates with TCF/LEF transcription factors, and TCF/LEF target genes become transiently activated (Moon et al., 2002).

We have recently discovered that Pitx2 gene, that encodes a transcription factor belonging to the bicoid family and exerts a crucial role during mammalian development (Gage et al., 1999), is a LEF1 target gene (Kioussi et al., 2002). We have described a Wnt/Dvl/β-catenin→Pitx2 pathway that mediates cell-type-specific proliferation during cardiac outflow tract and pituitary gland development. Once induced by Wnt signaling, Pitx2 is required for cell-type-specific proliferation and directly activates specific growth-regulating genes, such as Cyclin D1, Cyclin D2, and c-Myc (Kioussi et al., 2002, Baek et al., 2003). The rapid induction of Pitx2 by Wnt as well as the observation that the expression of Pitx2 is tightly regulated in time and space during development (Kioussi et al., 2002), led to the hypothesis that additional levels at which Pitx2 expression can be modulated might exist.

It is a rising concept that most genes are regulated by multiple mechanisms, the sum of which dictates the unique expression pattern of a gene under certain conditions. Several examples suggest that gene transcription and mRNA degradation rates are coordinately regulated to allow temporal modulation of gene expression (Proudfoot et al., 2002). The rate of mRNA turnover not only determines the rate of disappearance of mRNA but also its induction (Keene, 1999). mRNAs with short half-lives respond to changes in transcription more rapidly than those that are relatively stable, thus contributing to rapid changes in the pattern of cellular gene expression in response to changing environmental or developmental cues. Inherently unstable mRNAs include those encoding oncoproteins, cytokines, and cell cycle-regulated proteins (Chen and Shyu, 1995). Furthermore, during early Drosophila development, several genes undergo dramatic changes in abundance and in spatial distribution (Surdej et al., 1994). To achieve these rapid changes, multiple regulatory mechanisms exist which include both transcriptional control and regulation of mRNA processing (Surdej et al., 1994).

It has been previously shown that rapid degradation of mRNAs requires at least three components, (i) an instability element, such as the adenyate/uridyate-rich element (ARE) located in the 3′-untranslated region (UTR), (ii) certain ARE binding proteins (ARE-BPs), and (iii) an enzyme, the exosome. RNA decay is compromised by the removal of any of these components (Chen et al., 2001). AREs have been recognized as potent destabilizing elements in a wide variety of short-lived mRNAs (Chen and Shyu, 1995; Bakheet et al., 2001). AREs are grouped into three classes according to their...
sequence features and RNA decay characteristics (Chen and Shyu, 1995; Peng et al., 1996). Class I AREs contain 1 to 3 scattered copies of the pentanucleotide AUUUA embedded within a U-rich region, and are found in the c-Fos and c-Myc mRNAs. Class II AREs contain multiple overlapping copies of the AUUUA motif, and are found in cytokine mRNAs. Class III AREs, such as the one in c-Jun mRNA, lack the hallmark AUUUA pentanucleotide but present U-rich sequences. Some ARE-BPs have been proven to possess destabilizing activity on ARE-RNAs (TPP, BRF1, KSRP), while another (HuR) has been demonstrated to stabilize target transcripts (Blackshear, 2002; Stoecklin et al., 2002; Chen et al., 2001; R.G., M. Karin, and C.-Y.C, unpublished data; Brennan and Steitz, 2001). AUF1 has a dual role in ARE-mediated mRNA decay, functioning either as a destabilizing or a stabilizing factor depending on the cell type (Wilson and Brewer, 1999). The described ARE-BPs are required for regulation of class I and II ARE-RNAs. However, it is unclear whether the same ARE-BPs are involved in the control of class III ARE-RNAs. The exosome is a multi-subunit particle, containing nine 3'-to-5' exoribonucleases (Mitchell et al., 1997; van Hoof and Parker, 1999) and some ARE-BPs (Chen et al., 2001; Mukherjee et al., 2002), that rapidly degrades ARE-RNAs.

Here we report an unexpected mechanism controlling Pitx2 gene expression. Pitx2 mRNA is rapidly degraded due to the presence of destabilizing elements in both its coding sequence and 3' UTR. Activation of the Wnt/β-catenin pathway in pituitary cells induces a strong stabilization of Pitx2 mRNA as well as of Cyclins D1 and D2, and c-Jun mRNAs which are known to be transcriptional targets of the Wnt pathway (http://www.stanford.edu/~musse/wntwindow.html). Pitx2 mRNA stabilization correlates with a change in the pattern of interaction of both destabilizing and stabilizing ARE-BPs with Pitx2 3' UTR, with Pitx2 itself modulating the turnover of unstable mRNAs subsequent to Wnt/β-catenin activation.
RNA Decay Control by Wnt/β-Catenin

Figure 2. Pitx2 mRNA Is Stabilized Upon Activation of the Wnt/β-Catenin Signaling Pathway

(A) Stabilization of endogenous Pitx2 mRNA upon LiCl treatment in vivo. Serum-starved αT3-1 cells were treated with either 10 mM LiCl (Lithium) or water (Control) for 16 hr. Total RNA was isolated at the indicated times after addition of Actinomycin D and the levels of Pitx2 and β-actin mRNAs were determined by RNase protection.

(B) In vitro analysis of RNA decay upon LiCl treatment. Capped and polyadenylated RNAs (as indicated) were incubated with S100 prepared from either control or LiCl-treated αT3-1 cells and their decay analyzed by in vitro degradation assay as described in Figure 1C.

(C) Stabilization of ARE-containing RNAs upon stable expression of a constitutively active β-catenin (β-cateninc). S100 were prepared from either mock-transfected αT3-1 cells or αT3-1 stably expressing a constitutively active β-catenin (β-cateninc). RNA substrates (as indicated) were incubated with S100 and their degradation was examined as described in Figure 1C.

(D) Stabilization of Pitx2 3’UTR upon LiCl treatment does not depend on cap and polyadenylation. RNA substrates (as indicated) were incubated with either control or LiCl-treated αT3-1 and their decay was analyzed as described in Figure 1C. Arrowheads point to both polyadenylated and deadenylated forms of Pitx2 E2 p(A)+.

Results

Pitx2 mRNA Is Rapidly Degraded Due to the Presence of Destabilizing Sequences in Both Its Coding Region and Its 3’UTR

We have previously shown that Wnt activation rapidly induces Pitx2 expression both in embryos and in pituitary αT3-1 cells (Kioussi et al., 2002). Furthermore, Pitx2 mRNA levels rapidly decrease during C2C12 myoblasts differentiation (Kioussi et al., 2002). Pitx2 mRNA levels are high in the ectodermal primordium of the pituitary gland at E9.5–E10.5 while dramatically decrease at E12.5 (Figure 1A). Sustained in vivo overexpression of Pitx2 after E12.5 leads to hyperplasia of selected lineages in the anterior pituitary (Kioussi et al., 2002). Altogether these observations indicate that Pitx2 gene expression needs to be tightly regulated in time and space. We hypothesized that a posttranscriptional control of Pitx2 expression, additional to the transcriptional control, might exist.

To explore this possibility, we first measured the half-life of Pitx2 mRNA by RNase protection assay in pituitary αT3-1 cells at different times after Actinomycin D blockade of ongoing transcription. As shown in Figure 1B, Pitx2 mRNA was rapidly degraded with a half-life of approximately 40 min that is similar to that measured in vivo for cytokines, proto-oncogenes, and cell cycle-regulated proteins (Chen and Shyu, 1995).

A cell-free RNA decay system that reproduces several important aspects of regulated mRNA turnover in mammalian cells (Ford et al., 1999; Chen et al., 2001; Mukherjee et al., 2002) was used to identify the cis-acting sequences responsible for rapid degradation of Pitx2 mRNA. Among the three Pitx2 isoforms (Gage et al., 1999), we used in our experiments Pitx2a isoform (herein referred to as Pitx2). Internally 32P-labeled, capped, and polyadenylated RNA substrates synthesized in vitro induces Pitx2 expression both in embryos and in pituitary αT3-1 cells (Kioussi et al., 2002). Furthermore, Pitx2 mRNA levels rapidly decrease during C2C12 myoblasts differentiation (Kioussi et al., 2002). Pitx2 mRNA was as stable as GAPDH mRNA (Figure 1C). We analyzed Pitx2 3’UTR and found that it contains stretches of U-rich sequences resembling the class III (non-AUUUA) ARE described in c-Jun 3’UTR (Peng et al., 1996). To further define the sequences in the 3’UTR that confer instability to Pitx2 mRNA, we prepared RNA substrates including three distinct regions of Pitx2 3’UTR (E1, E2, and E3 in Figure 1D) and their decay rate was evaluated in vitro. As shown in Figure 1D, both E1 and E2 regions, that contain class III ARE, display destabilizing activity equivalent to that of the entire 3’UTR. On the contrary, the transcript E3 which does not include ARE was stable in vitro (Figure 1D).

We and other groups previously showed that rapid degradation of class I and II ARE-RNAs requires the
exosome (Chen et al., 2001; Mukherjee et al., 2002). Therefore, we examined whether the class III ARE-containing Pitx2 3’UTR RNA was degraded by the exosome. We removed the exosome from αT3-1 cells S100 by immunodepletion using the anti-PM-Scl serum, as judged by the complete removal of the core component Rrp4p (Chen et al., 2001) (Figure 1E, left panel), and compared the decay activity of these extracts with control-immunodepleted extracts. As presented in Figure 1E (right panel), exosome removal strongly stabilizes Pitx2 3’UTR-containing mRNA.

Altogether these data suggest that the expression of Pitx2 can be regulated at the level of its transcript turnover. Two regions, the CDS and the 3’UTR, contain destabilizing sequences. Pitx2 3’UTR displays class III ARE mediating exosome-dependent mRNA degradation.

Stabilization of Pitx2 mRNA Upon Activation of the Wnt/β-Catenin Signaling Pathway

It is well known that activation of the Wnt/β-catenin signaling pathway induces profound changes in the expression of target genes through regulation of the TCF/LEF transcription factors (Moon et al., 2002). However, it is unknown whether Wnt/β-catenin signaling is involved in posttranscriptional control of gene expression. We investigated whether Wnt/β-catenin activation affects the turnover rate of Pitx2 mRNA. We showed that Wnt activation, mimicked by lithium chloride (LiCl) (Hedgepeth et al., 1997), stabilizes Pitx2 mRNA in mouse

Figure 3. Wnt Activation Affects Both the Cytoplasmic Levels and the Interaction of Destabilizing and Stabilizing ARE-BPs with RNA (A) Specific interaction of ARE-BPs with Pitx2 3’UTR in vitro. Either different concentrations (50, 100, or 200 ng) of purified recombinant KSRP, TTP, BRF1, AUF1, or HuR, in the absence of any competitor RNA (lanes 1–3), or 100 ng of each recombinant protein, in the presence of a 50-fold excess of either unlabelled Pitx2 3’UTR RNA (lane 4) or unlabelled Pitx2 E3 RNA (lane 5), were incubated with 32P-labeled Pitx2 3’UTR RNA in a 20 μl reaction volume and UV-crosslinking assays were performed. (B) Interaction between KSRP, TTP, or HuR and Pitx2 3’UTR in control or LiCl-treated S100. S100 from either untreated (“C”) or LiCl-treated cells (“LiCl”) were incubated with Pitx2 3’UTR RNA. After UV-crosslinking, the reactions were immunoprecipitated with control antibodies (preimmune sera or control IgG) or αKSRP, αTTP, or αHuR antibodies as indicated, separated by SDS-PAGE, and autoradiographed (top panel). Immunoprecipitates presented in the top panel were analyzed by immunoblot analysis using αKSRP (lanes 1–3), αTTP (lanes 4–6), or αHuR (lanes 7–9; bottom panel). (C) In vivo association of Pitx2 mRNA with TTP, KSRP, and HuR. After formaldehyde fixation of either control or LiCl-treated intact αT3-1 cells, the proteins were immunoprecipitated from cell extracts with the indicated antibodies. RNA was extracted from the immune complexes and analyzed by RT-PCR. (D) LiCl treatment does not affect the expression of KSRP, TTP, and HuR. Total cell lysates were prepared from either control or LiCl-treated cells; aliquots were analyzed by immunoblot with the indicated antibodies. (E) Nucleocytoplasmic transport of KSRP, TTP, and HuR upon LiCl treatment. Nuclear (lanes 1 and 2) or cytoplasmic (lanes 2 and 3) extracts were prepared from either control or LiCl-treated αT3-1 cells and analyzed by immunoblot using αKSRP, αTTP, αHuR, anti-β-tubulin, and αHDAC2 antibodies.
pituitary αT3-1 cells increasing its half-life from 40 min to more than 120 min (Figure 2A). By in vitro degradation assay, we observed that the entire Pitx2 RNA was unstable in S100 from untreated cells, while it was more than 3-fold stabilized in extracts from LiCl-treated cells (Figure 2B). Similar stabilization upon LiCl treatment was also observed with the 3′UTR, E1, and E2 RNA substrates (Figure 2B). In contrast, the instability element found in Pitx2 CDS was unresponsive to LiCl treatment. No significant difference in the stability of stable GAPDH and E3 RNA substrates was observed in the S100 from either cells.

LiCl does not mimic exclusively the Wnt signaling activation (Phiel and Klein, 2001). To verify whether the canonical Wnt/β-catenin pathway is responsible for the LiCl-induced stabilization of Pitx2 mRNA we analyzed S100 from αT3-1 cells expressing a constitutively active form of β-catenin (β-cat-enin) that exhibits nuclear localization in the absence of Wnt signals (Klöussi et al., 2002). Similarly to LiCl treatment, expression of β-catenin, stabilizes Pitx2 3′UTR RNA without affecting the stability of Pitx2 CDS (Figure 2C). These findings suggest that the destabilizing elements in this region are not involved in Wnt-dependent Pitx2 mRNA stabilization. Interestingly, Wnt activation does not affect the stability of other ARE-containing unstable mRNAs including TNFα (Figures 2B and 2C), IL-4, and IL-2 (data not shown).

The earliest event in the decay of most mRNAs is shortening of the poly(A) tail. Once this step is complete, the 5′-7-methylguanosine cap is removed and, finally, the mRNA body is rapidly degraded (Wilson et al., 2001). To investigate whether Pitx2 mRNA decay is controlled by Wnt activation at the level of either decapping or deadenylation, we analyzed the decay rate of two synthetic RNAs containing Pitx2 3′ UTR lacking either CAP or poly(A) tail. As shown in Figure 2D, both RNAs are stabilized by Wnt activation in a way superimposable to polyadenylated and capped Pitx2 3′ UTR mRNA. Furthermore, LiCl treatment stabilizes the polyadenylated Pitx2 E2 RNA without affecting its deadenylation rate (Figure 2D). Altogether our findings suggest that the molecular target of Wnt signaling is the ARE-mediated decay of the mRNA body.

The evidence that Pitx2 3′ UTR RNA, containing class III ARE, is stabilized by Wnt pathway activation while TNFα, IL-4, and IL-2 AREs (class I/II ARE) are not, raises the possibility that Wnt activation induces a class III ARE-specific mRNA stabilization. To verify this hypothesis, we analyzed in vitro the decay rate of additional ARE-containing mRNAs. c-Jun ARE and Cyclin D2 ARE belong to class III, while Cyclin D1 ARE belongs to class I. Cyclin D2 mRNA has not previously reported to contain destabilizing sequences in its 3′ UTR. Immunodepletion experiments indicated that Cyclin D2 ARE-directed mRNA rapid decay is exosome mediated and requires the interaction between ARE and ARE-BPs (data not shown). Results presented in Figures 2B and 2C indicate that all three RNA substrates are stabilized by either LiCl treatment or β-catenin expression. These results rule out the possibility that a class III ARE-restricted effect upon Wnt activation exists. Interestingly, Pitx2, c-Jun, Cyclin D1, and Cyclin D2 are all transcriptional targets of the Wnt signaling pathway (http://www.stanford.edu/~rmussl/wntwindow.html). Therefore, this may suggest the existence of a specific regulatory code that conveys a unique signal into both transcriptional and mRNA turnover changes, to coordinately regulate multiple genes.

**ARE-BPs Interplay Is Involved in Wnt-Induced mRNA Stabilization**

We investigated whether any of the known ARE-BPs are involved in mRNA decay mediated by the class III ARE found in Pitx2 3′ UTR and whether changes in the activity
of these ARE-BPs are able to confer mRNA stabilization in response to Wnt activation.

First, we examined the ability of recombinant ARE-BPs, including KSRP, TTP, BRF1, AUF1, and HuR, to interact with Pitx2 3'UTR in vitro. As shown in Figure 3A, all the proteins display a concentration-dependent binding to Pitx2 3'UTR RNA in a UV-crosslinking assay. The binding of the recombinant ARE-BPs to Pitx2 3'UTR was efficiently competed by unlabeled Pitx2 3'UTR, but not by Pitx2 E3 RNA (Figure 3A, lanes 4 and 5). Next, we investigated whether Wnt/β-catenin activation induces changes in the interaction between endogenous ARE-BPs and Pitx2 3'UTR. In the experiments shown in Figure 3B, labeled Pitx2 3'UTR was incubated with S100 prepared from either untreated or LiCl-stimulated αT3-1 cells. After UV crosslinking, samples were immunoprecipitated with either control or specific anti-ARE-BPs antibodies. LiCl dramatically decreased both KSRP and TTP interaction with Pitx2 3'UTR, while it strongly increased HuR-Pitx2 3'UTR binding (Figure 3B, top panel). Immunoblot analysis of the immunoprecipitates indicates that the RNA binding activity parallels the amount of ARE-BPs immunoprecipitated from either control or LiCl-treated S100 (Figure 3B, bottom panel). We failed to observe an interaction of the endogenous AUF1 with Pitx2 3'UTR RNA in vitro (data not shown).

To investigate the in vivo interaction between ARE-BPs and Pitx2 mRNA, we immunoprecipitated lysates from either untreated or LiCl-stimulated formaldehyde-fixed αT3-1 cells, according to the procedure described by Nirajanakumari et al. (2002) (RNA immunoprecipitation). The coimmunoprecipitated RNAs were reverse transcribed and the presence of Pitx2 mRNA in the immunoprecipitates was analyzed by PCR using sequence-specific primers. As shown in Figure 3C, both anti-TTP and anti-KSRP immunoprecipitated Pitx2 mRNA from untreated cell lysates while anti-HuR failed to immunoprecipitate the transcript. On the contrary, after LiCl treatment, Pitx2 mRNA was present in anti-HuR immunoprecipitates while it was absent from both anti-TTP and anti-KSRP immunoprecipitates (Figure 3C). Similar results were obtained in β-catenin-expressing αT3-1 cells (data not shown).

We investigated whether the difference in the amount of KSRP, TTP, and HuR detected in S100 upon LiCl-treatment results from changes in their expression levels. Total lysates were prepared from either control or LiCl-treated αT3-1 cells and subjected to immunoblot with specific antibodies. As shown in Figure 3D, no significant changes in the total amount of KSRP, TTP, and HuR was detected in LiCl-treated cells compared to control cells. Therefore, we examined whether changes in the nucleocytoplasmic shuttling of KSRP, TTP, and HuR could account for the difference in the amount of the proteins immunoprecipitated from S100 upon LiCl treatment. Cytoplasmic and nuclear fractions were prepared from control or LiCl-treated αT3-1 cells and analyzed by immunoblot. A predominantly nuclear protein (HDAC2) is present exclusively in nuclear extracts while a predominantly cytoplasmic protein (β-tubulin) is present only in cytoplasmic extracts of αT3-1 cells, thus demonstrating the effectiveness of our fractionation procedure. KSRP and TTP are present in both nucleus and cytoplasm of several cell lines including αT3-1 (Figure 3E). LiCl treatment caused a strong reduction in the cytoplasmic pools of KSRP and TTP while increased their levels in the nucleus (Figure 3E). In αT3-1 cells, HuR is predominantly nuclear. LiCl treatment of αT3-1 cells caused a strong increase of HuR cytoplasmic levels and a decrease of nuclear levels (Figure 3E). Altogether these data suggest that Wnt activation induces changes in the cytoplasmic distribution of KSRP, TTP, and HuR, leading to changes in Pitx2 mRNA-ARE-BPs interactions that, in turn, can be responsible for Pitx2 mRNA stabilization.

To explore the possibility that the described changes in ARE-BPs binding to Pitx2 mRNA are responsible for LiCl-induced mRNA stabilization, we investigated by in vitro degradation assays whether ARE-BPs are involved in the Wnt-dependent mRNA stabilization. Recombinant purified TTP, KSRP, BRF1, and AUF1 were added to S100 from LiCl-treated αT3-1 cells. All the proteins, but AUF1, induced a destabilization of the LiCl-stabilized Pitx2 3'UTR RNA over a concentration range from 25 to 500 ng reaching a plateau effect between 50 and 100 ng (data not shown). When used at the fixed amount of 50 ng, TTP, KSRP, and BRF1 destabilized, in a time-dependent manner, Pitx2 3'UTR, c-Jun ARE, and Cyclin D1 ARE mRNAs which are stable when incubated with S100 from LiCl-treated αT3-1 cells (Figure 4A). Because AUF1 has a dual, cell-type-dependent role on mRNA stability and it interacts with Pitx2 3'UTR in vitro, we investigated whether recombinant AUF1 protects from degradation the Pitx2 mRNA unstable in the presence of control αT3-1 extracts. At concentrations ranging from 25 to 1000 ng, recombinant AUF1 was unable to affect Pitx2 3'UTR mRNA stability (data not shown). We next examined whether addition of a stabilizing ARE-BP, HuR, to the S100 from untreated αT3-1 cells protects Pitx2 3'UTR RNA from degradation. Addition of recombinant HuR stabilizes Pitx2 3'UTR, c-Jun, and Cyclin D1 ARE mRNAs at relatively high concentrations (200 ng, Figure 4A). The observation of HuR-mediated RNA stabilization and of the increased HuR cytoplasmic levels upon Wnt activation, prompted us to examine whether HuR is required for Wnt-induced Pitx2 mRNA stabilization. HuR was removed from LiCl-treated αT3-1 S100 by immunodepletion (Figure 4B, left panel). As presented in the right panel of Figure 4B, HuR removal accelerated the decay rate of Pitx2 3'UTR RNA. Importantly, the addition of recombinant HuR to immunodepleted extracts restored the stability of Pitx2 3'UTR RNA observed in control IgG-immunodepleted extracts (Figure 4B, right panel). The stability of GAPDH RNA was not affected by any treatments (Figures 4A and 4B). Our data indicate that both stabilizing and destabilizing ARE-BPs are coordinately involved in Wnt-dependent stabilization of mRNA molecules encoded by Wnt target genes.

Pitx2 Plays a Central Role in Wnt-Induced mRNA Stabilization

Because Pitx2 expression is strongly induced by Wnt signaling and Pitx2, in turn, plays a central role in regulating the expression of downstream targets of this pathway (Kioussi et al., 2002), we investigated whether Pitx2 itself could be involved in the Wnt-induced stabilization
Figure 5. Pitx2 Plays a Central Role in Wnt-Induced RNA Stabilization

(A) Expression of tagged Pitx2 and mHDPitx2 in αT3-1 cells. Total extracts of mock-transfected αT3-1 cells and αT3-1 expressing either CBP-Pitx2 or CBP-mHDPitx2 were subjected to immunoblot analysis using αPitx2. Asterisk marks the position of the tagged Pitx2 proteins while arrowheads point to the isoforms of endogenous Pitx2.

(B) Stabilization of ARE-RNA substrates by overexpression of Pitx2. RNA substrates including Pitx2 3'UTR, c-Jun, Cyclin D1 and D2 AREs, or GAPDH were incubated with S100 prepared from either untreated or LiCl-treated mock-transfected αT3-1 cells and αT3-1 cells stably expressing either wild-type Pitx2 (Pitx2) or mHDPitx2 (mHD). RNA decay was examined by the in vitro degradation assay.

(C) LiCl treatment strongly increases cytoplasmic Pitx2 levels. Cytoplasmic or nuclear extracts were prepared from either control or LiCl-treated αT3-1 cells and analyzed by immunoblotting using αPitx2.

(D) HuR coimmunoprecipitates with Pitx2. (Left panel) S100 from LiCl-treated αT3-1 cells were immunoprecipitated with cIgG or αHuR antibody. The precipitates were subjected to immunoblot using αPitx2. 10% input of S100 was also included. (Right panel) S100 from LiCl-treated αT3-1 cells were incubated with 32P-labeled c-JunARE RNA and subjected to UV crosslinking followed by immunoprecipitation using αHuR, αPitx2, or preimmune sera, blotted to a nylon membrane, and autoradiographed (top panel). The same membrane was reacted with αHuR (bottom panel).

(E) Interaction between recombinant purified HuR and Pitx2. GST pull-down experiments were performed using bacterially expressed GST or GST-HuR proteins and baculovirus-expressed purified Pitx2. Pitx2 was revealed by immunoblotting using αPitx2. 10% input of purified Pitx2 was also included.

(F) HuR interacts with wild-type Pitx2 but not with mHDPitx2. Cytoplasmic extracts were prepared from LiCl-treated Pitx2αT3-1 and mHDαT3-1 cells, precipitated with calmodulin-Sepharose beads, and analyzed by immunoblotting using αHuR. 10% input of the unprecipitated S100 was included.

(G) Pitx2 is required for stabilization of RNA in LiCl-treated S100. (Left panel) S100 depleted with preimmune or αPitx2 sera were analyzed by immunoblotting using the indicated antibodies. (Right panel) Pitx2 3'UTR RNA was incubated with either preimmune- or αPitx2-depleted S100 and its decay was analyzed. In some experiments, recombinant GST (50 ng) or Pitx2 (50 ng) or mHDPitx2 (50 ng) were added to the in vitro degradation reaction (25 μl).
of Pitx2, c-Jun, Cyclin D1, and Cyclin D2 ARE-RNAs. Either a wild-type Pitx2 or a Pitx2 mutated in the homeo-domain (mHDPitx2) (Kioussi et al., 2002) were stably expressed at the same levels in αT3-1 cells (Pitx2:αT3-1 and mHDαT3-1, respectively) (Figure 5A). Pitx2 antibody recognizes two isoforms of endogenous Pitx2 (arrowheads in Figure 5A). We previously reported that mHDPitx2 is unable to exert Pitx2 effects on cell proliferation control (Kioussi et al., 2002). S100 were prepared from both cell lines under either basal conditions or after LiCl treatment, and the stability of different ARE-containing mRNA substrates was estimated in vitro. Whereas Pitx2 3’UTR, c-Jun, Cyclin D1, and Cyclin D2 ARE RNAs were unstable in the S100 from untreated mock-transfected αT3-1 cells and mHDαT3-1 cells, these RNAs were significantly stabilized in the S100 from untreated mock-transfected αT3-1 cells and mHDαT3-1 cells (Figure 5B). The stabilization of these RNAs in Pitx2αT3-1 cells was similar to that observed in LiCl-treated mock-transfected αT3-1 cells. Furthermore, expression of mHDPitx2 inhibited the LiCl-induced mRNA stabilization (Figure 5B). These data suggest that Pitx2 is involved in mRNA stabilization induced by Wnt activation and that a functional homeodomain is a required component of the Wnt-induced mRNA stabilization of a selected set of transcripts.

To further investigate Pitx2 role in Wnt-induced mRNA stabilization, we examined the expression of Pitx2 upon LiCl treatment. Cytoplasmic and nuclear fractions were prepared from either control or LiCl-treated αT3-1 cells and subjected to immunoblot analysis. Pitx2 was present at low levels in the cytoplasm of αT3-1 cells, and LiCl treatment strongly increased the levels of cytoplasmic Pitx2 (Figure 5C). As expected (Kioussi et al., 2002), the total levels of Pitx2 were increased by LiCl treatment (data not shown). Preliminary results from our laboratory indicate that Pitx2 belongs to a large multiprotein complex comprising some RNA binding proteins (P.B., C.I., P. Tempst, and M.G.R., unpublished data). Therefore, we investigated whether Pitx2 can interact with HuR, and we found that Pitx2 and HuR communoprecipitate in S100 of LiCl-stimulated αT3-1 cells (Figure 5D, left panel). To further investigate Pitx2/HuR interactions, labeled c-Jun ARE mRNA was incubated with S100 from LiCl-treated αT3-1 cells. After UV crosslinking, samples were immunoprecipitated with αHuR, αPitx2, or preimmune serum. As shown in Figure 5D (right panel), an RNA-bound protein corresponding to HuR (see immunoblot at the bottom of the panel) was detected in αPitx2 immunoprecipitants. However, the interaction between Pitx2 and HuR does not require HuR binding to RNA, as demonstrated by GST pull-down experiments shown in Figure 2E. Next, we investigated Pitx2/HuR interactions in Pitx2αT3-1 and mHDαT3-1 cells expressing either calmodulin binding peptide (CBP)-tagged wild-type Pitx2 or CBP-tagged mHDPitx2, respectively. Cytoplasmic extracts were subjected to precipitation using calmodulin beads and analyzed by αHuR in immunoblotting. The wild-type Pitx2, but not mHDPitx2, coprecipitated HuR (Figure 5F).

To provide a functional significance to the Pitx2-HuR interaction in stabilization of mRNA, Pitx2 was removed from LiCl-treated S100 (Figure 5G, left panel) and the degradation of Pitx2 3’UTR RNA was examined. The RNA was stable in preimmune serum-depleted S100 from LiCl-treated αT3-1 cells, while the stabilization was inhibited in αPitx2-depleted S100 (Figure 5G). Recombinant Pitx2 reconstituted RNA stabilization when added to αPitx2-depleted extracts while recombinant mHDPitx2 was ineffective (Figure 5G, right panel). Pitx2 immunodepletion removed only a portion (less than 50%) of HuR present in S100 (Figure 5G, left panel).

These data indicate that Pitx2 plays a central role in the Wnt-activated regulatory pathway that stabilizes Pitx2, Cyclin D1, Cyclin D2, and c-Jun RNAs. Pitx2 effect is mediated, in part, by its interaction with HuR.

Discussion

Regulation of mRNA Stability by the Wnt/β-Catenin Pathway

In this manuscript we show that the mRNA encoded by Pitx2, a transcriptional target and an effector molecule of the Wnt/β-catenin pathway, is unstable both in vitro and in vivo. The activation of the Wnt/β-catenin signaling pathway stabilizes Pitx2 mRNA by targeting the ARE present in its 3’UTR. Importantly, Wnt/β-catenin activation causes stabilization of three ARE-containing labile mRNAs encoded by three additional transcriptional target genes of the same pathway, c-Jun, Cyclin D1, and Cyclin D2. Upon Wnt activation, the cytoplasmic levels and the mRNA interaction of the two destabilizing ARE-BPs, KSRP and TTP, are reduced while the cytoplasmic levels and the mRNA interaction of HuR, a stabilizing ARE-BP, strongly increase. In vitro reconstitution/depletion experiments suggest that the combined regulation of these three ARE-BPs recapitulates Wnt/β-catenin-dependent mRNA stabilization. Importantly, Pitx2 itself is involved in the Wnt-dependent mRNA turnover control.

It has become clear in the recent few years that gene expression in eukaryotes requires several multicomponent cellular machines. Recent studies lead to the view that a complex and extensively coupled network has evolved to coordinate the activities of the gene expression machines. For instance, when required, the transient activation of gene transcription can be converted
into a prolonged expression by modulation of transcript half-life (Orphanides and Reinberg, 2002). During organogenesis, mRNA turnover control is supposed to play a relevant role in conveying developmental cues in rapid fluctuations of transcripts coding for key regulators of proliferative/differentiative events (Surdej et al., 1994). Notably, an ARE-dependent control of the turnover of labile transcripts encoded by genes regulating mammalian organogenesis has not been reported yet.

While much knowledge has accumulated regarding the mechanisms by which signal transduction pathways regulate gene expression at the transcriptional level, it has become evident in the last years that the same pathways have dramatic effects on mRNA stability control. Activation of signaling pathways including JNK, MAPK p38, PI3K, ERK, and cAMP-activated kinase has been proven to induce relevant changes in the turnover rate of unstable mRNAs coding for cytokines, proto-oncogenes, cell cycle regulatory proteins, and growth factors (Chen et al., 2000; Ming et al., 2001; Westmark and Malter, 2001; Wang et al., 2002). The knowledge that Wnt activation is involved in both embryonic development and cancer led to a large number of investigations focused on the transcriptional control of the expression of Wnt target genes (reviewed in Cadigan and Nusse, 1997; Moon et al., 2002). Our data provide evidence that Wnt/β-catenin pathway activation can induce profound changes in mRNA turnover as well. Moreover, results presented here points to ARE and ARE-BPs as final targets of Wnt/β-catenin activation.

**Wnt/β-Catenin Regulates ARE Binding Protein Function and Subcellular Localization**

We show that both the in vitro and the in vivo binding activity of endogenous KSRP and TTP to Pitx2 mRNA is strongly reduced upon Wnt activation, while the binding activity of HuR to Pitx2 mRNA is increased. These changes in ARE-BPs/mRNA interactions reflect Wnt activation-dependent changes in the cytoplasmic levels of the same ARE-BPs. Conspicious experimental evidences both in vitro and in vivo indicate that TTP is a destabilizing ARE-BP (reviewed in Blackshear, 2002). Also KSRP, a novel ARE-BP that copurifies with the exosome, is required for rapid decay of unstable mRNAs both in vitro and in vivo (Chen et al., 2001; R.G., M. Karin, and C-Y.C., unpublished data). In vitro reconstitution experiments indicate that, under conditions of Wnt signaling activation, the addition of recombinant KSRP and TTP to the cell-free system restores the rapid decay of Pitx2, c-Jun, Cyclin D1, and Cyclin D2 mRNAs. This suggests that the decreased interaction of mRNAs with these destabilizing ARE-BPs is a major event that leads to mRNA stabilization upon Wnt activation. Accordingly, KSRP overexpression in αT3-1 cells strongly reduces the LiCl-induced mRNA stabilization (P.B. and R.G., unpublished data).

It is now clear that HuR subcellular localization is intimately linked to its stabilizing function (reviewed in Brennan and Steitz, 2001). HuR is predominantly (>90%) localized in the nuclei of unstimulated cells and it translocates to the cytoplasm of stimulated cells in order to exert its mRNA-stabilizing activity (Gallozzi et al., 2000; Wang et al., 2000, 2002). Indeed, Wnt activation in αT3-1 cells strongly increases both HuR cytoplasmic levels and its interaction with mRNA. Furthermore, reconstitution/depletion experiments indicate that HuR is required for stabilization of Pitx2, c-Jun, Cyclin D1, and Cyclin D2RNAs. Our in vitro results suggest that other ARE-BPs could be involved in this complex regulatory event. BRF1 is as efficient as either KSRP or TTP in reconstituting rapid mRNA decay when added to S100 prepared from LiCl-treated cells. In light of the observation that Wnt activation does not affect ARE-BPs expression levels, it is tempting to speculate that the effect on mRNA stability induced by Wnt/β-catenin activation is caused, at least in part, by regulated and coordinated cytoplasmic translocation of both destabilizing and stabilizing ARE-BPs.

**Pitx2 as a Target and Mediator of Wnt/β-Catenin Regulated mRNA Stability**

We propose a model that integrates our view of how Wnt signaling can regulate the expression of target genes in pituitary-derived cells (Figure 6). Wnt activation rapidly induces, through LEF1, the transcription of Pitx2 and of additional target genes, including c-Jun, Cyclin D1, and Cyclin D2, through either TCF/LEF or Pitx2 (Mann et al., 1999; Kioussi et al., 2002). Here we report that Wnt activation regulates the expression of the same target genes affecting their mRNA turnover rates. Once induced, Pitx2 itself plays a central role in the stabilization of its own transcript and in the turnover control of other unstable transcripts.

Our results suggest a direct role of Pitx2 in controlling HuR function. First, LiCl treatment of αT3-1 cells strongly increases cytoplasmic levels of Pitx2. Second, Pitx2 interacts with HuR. Third, a Pitx2 mutant unable to exert cell-type-specific proliferation control (Kioussi et al., 2002) does not interact with HuR and does not reconstitute RNA stabilization in cPitx2-immunodepleted S100. Furthermore, this mutant blocks LiCl effect on mRNA stability functioning as a Pitx2 dominant negative in αT3-1 cells. Fourth, neither recombinant nor endogenous Pitx2 is able to directly interact with ARE mRNAs (data not shown), ruling out the possibility of an ARE binding-dependent function of Pitx2 in mRNA stabilization. It is tempting to speculate that Pitx2 plays a role in modulating the cytoplasmic concentration of HuR and, consequently, its in vitro and in vivo binding activity to ARE-mRNAs. However, on the basis of our data, we suggest that Pitx2 regulates additional events that control mRNA stability. Pitx2 immunodepletion from LiCl-treated S100 removes less than 50% of HuR present in the extracts while it causes a complete destabilization of Pitx2 3’ UTR RNA. Conversely, complete HuR immunodepletion from LiCl-treated S100 (that removes less than 50% of Pitx2 [data not shown]) does not completely destabilize Pitx2 3’ UTR RNA. Altogether these results suggest that HuR is not the only target of Pitx2-mediated mRNA stabilization.

**Conclusions**

Regulating a rate-limiting step is an efficient way to control the overall rate of a multistep process. However, there is a limit to the level of regulation that can be achieved by controlling a single step; there is no point
in increasing the rate of one step if another soon becomes rate limiting. Therefore, eukaryotes have developed methods to regulate the expression of their proteins at multiple levels in a coordinated fashion. The mechanisms used largely depend on the level of regulation required for proper gene function and are selected through evolution. Genes whose expression must be rapidly and tightly controlled tend to be quickly transcribed and translated, and their mRNAs and proteins have short half-lives.

Our data add a further level to the definition of a multistep regulation by the Wnt/β-catenin pathway that enhances the expression of selected genes by, at least, two independent and coordinated mechanisms. In a sense, these events convert Pitx2 from a rapidly induced gene to a more stable regulator of cell-type-specific gene function.

Experimental Procedures

Cell Lines

γT3-1 cells, mock transfected, and β-catenin, stably transfected γT3-1 cells have been previously described (Kioussi et al., 2002). PCR-amplified cDNAs coding for Pitx2a and for mutated Pitx2a (bearing three amino acid substitutions within the homeodomain [Kioussi et al., 2002]) were cloned in the the KpnI-EcoRI sites of pcDNA3-TAP (Chen et al., 2001) in-frame with the coding sequence of the IgG binding domain of Protein A and the calmodulin binding peptide (CBP) obtaining CBP-Pitx2 and CBP-mHDPitx2, respectively. γT3-1 cells were transfected with pcDNA3-TAP (control), CBP-Pitx2, or CBP-mHDPitx2 by Lipofectamine plus (Invitrogen), and after 48 hr, cells were cultured in selective medium (500 µg/ml G418). Cells were used for experiments. To perform LiCl treatment, both γT3-1 cells and stable transfectants exponentially growing were serum starved for 24 hr, then incubated overnight with either 10 mM LiCl (dissolved in water) or water.

Plasmids, In Vitro Degradation, and UV Crosslinking

cDNA fragments containing the entire Pitx2a isoform cDNA, 250 bp of Pitx2a 3’UTR, Pitx2a coding sequence (CDS), the entire Pitx2a 3’UTR (or its deletion fragments as shown in Figure 1D), and the ARE of Cyclin D1 (2410–2643 nt), Cyclin D2 (5880–6170 nt), TNFα/H11032 were cloned in the KpnI-EcoRI sites of pcDNA3-TAP (Chen et al., 2001) in-frame with the coding sequence of the IgG binding domain of Protein A and the calmodulin binding peptide (CBP) obtaining CBP-Pitx2 and CBP-mHDPitx2, respectively. γT3-1 cells were transfected with pcDNA3-TAP (control), CBP-Pitx2, or CBP-mHDPitx2 by Lipofectamine plus (Invitrogen), and after 48 hr, cells were cultured in selective medium (500 µg/ml G418). Cells were used for experiments. To perform LiCl treatment, both γT3-1 cells and stable transfectants exponentially growing were serum starved for 24 hr, then incubated overnight with either 10 mM LiCl (dissolved in water) or water.

Recombinant Proteins and Antibodies

GST- and histidine-tagged proteins were produced in BL21(DE3) cells and purified on Glutathione-Sepharose 4B (Pharmacia Biotech) and Ni-NTA (Qiagen) resins, respectively, as previously described (Chen et al., 2001; Ming et al., 2001; Stoecklin et al., 2002), Histidine-tagged KSRP was previously described (Chen et al., 2001). The use of anti-Rp4p and autoimmune sera from PM-Scl patients was previously described (Chen et al., 2001). A polyclonal antibody to KSRP was raised in rabbit, γT3-1 cells was a guinea pig polyclonal antibody (Kioussi et al., 2002), and γHu monotonic antibody 19F12 was from Molecular Probes Inc. Rabbit polyclonal γTTP (H-120) and monoclonal γHDAC2 antibodies were from Santa Cruz Inc. Monoclonal antibody to β-tubulin was from Sigma.

RNA Immunoprecipitation

RNA immunoprecipitation (RIP) was performed according to the method described by Niranjanakumari et al. (2002). Briefly, both control and LiCl-treated cells were trypsinized, extensively washed, and incubated 10 min at room temperature with 0.38 M formaldehyde. Crosslinking was quenched, cells were extensively washed, and lysed by sonication in RIPA buffer. Aliquots of the precleared lysates were immunoprecipitated with Protein A-Sepharose-bound antibodies at 4°C overnight. The beads were washed six times in RIPA buffer containing 3 M urea, crosslinking was reversed by incubating samples 45 min at 70°C and RNA was extracted. RNA aliquots were subjected to reverse transcription and PCR using primers specific for either Pitx2a or β-actin sequences (30 cycles using an annealing temperature of 60°C).

Immunodepletion Experiments

Specific proteins were removed from S100 by four 4 hr rounds of immunoprecipitation at 4°C with antibodies prebound to Protein A-Sepharose. Equal amounts of supernatants from control serum and antiserum-depleted reactions were analyzed by immunoblotting and subsequently used in RNA decay assays.

In Situ Hybridization and RNase Protection

In situ hybridization was performed as previously described (Simmons et al., 1990). Exponentially growing γT3-1 cells were serum starved for 24 hr and treated with either 10 mM LiCl (lithium) or water (control) for 16 hr. Then cells were treated with 5 µg/ml Actinomycin D, harvested at the indicated times, and total RNA was prepared by RNAzol (Tel-Test Inc.). Pitx2 and β-actin mRNA levels were analyzed by RNase protection assay using the RPA III kit (Ambion).

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