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## p120-catenin regulates REST and CoREST, and modulates mouse embryonic stem cell differentiation

moonsup lee

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**p120-catenin regulates REST and CoREST, and modulates mouse embryonic  
stem cell differentiation  
by**

**Moonsup Lee, M.S.**

---

**Pierre D. McCrea, Ph.D.  
Supervisory Professor**

---

**Jae-il Park, Ph.D.**

---

**Xiaobing Shi, Ph.D.**

---

**Zhimin Lu, M.D., Ph.D.**

---

**Amy K. Sater, Ph.D.**

**APPROVED:**

---

**Dean  
The University of Texas  
Health Science Center at Houston  
Graduate School of Biomedical Sciences**

**p120-catenin regulates REST and CoREST, and modulates mouse embryonic  
stem cell differentiation**

**A**

**DISSERTATION**

**Presented to the Faculty of**

**The University of Texas**

**Health Science Center at Houston**

**And**

**The University of Texas**

**M.D. Anderson Cancer Center**

**Graduate School of Biomedical Sciences**

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**of the Requirements**

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**by**

**Moonsup Lee, M.S.**

**Houston, Texas**

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# **p120-catenin regulates REST and CoREST, and modulates mouse embryonic stem cell differentiation**

Moonsup Lee, Ph.D.

Advisory Professor: Pierre D. McCrea, PhD.

The canonical-Wnt pathway and beta-catenin have been extensively studied to determine their contributions to stem cell biology, but less is known about p120-catenin in the nuclear compartment. P120 is developmentally required as a consequence of its biochemical and functional interactions with cadherins, small-GTPases and transcriptional regulators. We report here that p120-catenin binds to and negatively regulates REST and CoREST, that others have indicated form a repressive complex having diverse key roles in developmental and pathologic gene regulation. We thus provide the first evidence for a direct upstream modulator of REST/CoREST function. Using mouse embryonic stem cells (mESCs), mammalian cell lines, *Xenopus* embryos, and in vitro systems, we show that p120 directly binds to the zinc finger/DNA-binding region of REST, as well as to CoREST. Chromatin immuno-precipitation and other approaches indicate that p120 protein levels negatively determine the extent of REST/CoREST bound to RE1 consensus binding sites and negatively influence REST/CoREST protein stability. As would be predicted, p120 overexpression and depletion have complementary effects upon REST/CoREST gene-target activity. Thus, p120 depletion in mESCs reduces REST/CoREST gene-target expression, while p120 overexpression has a converse effect. Importantly, p120 levels modulate the mRNA and protein levels of Oct4,

Nanog, and Sox2, and have an impact upon the differentiation of mESCs towards neural fates. In assessing potential upstream inputs of this novel p120-REST/CoREST pathway, REST gene targets were found to respond to the level of E-cadherin, with cadherin effects being dependent on p120-catenin as predicted. In summary, at both biochemical and functional levels, our findings reveal a central role of p120-catenin in the derepression (activation) of genes directly controlled by REST/CoREST, and in the modulation of stem cells.

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# **CHAPTER I**

## **INTRODUCTION**

## *Pluripotency and Differentiation of mouse embryonic stem cells (mESCs)*

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of the developing embryo blastocyst. mESCs are capable of differentiating into all cell lineages (pluripotency), as well as generating the same state of daughter cells resulting from a cell division (self-renewal). In the absence of factors maintaining stemness, such as Leukemia Inhibitory Factor (LIF) in culture, mESCs differentiate into different lineage cells in a context dependent manner.

The pluripotency and lineage-specific differentiation pathways are tightly modulated by multiple intrinsic and extrinsic signals that are transmitted through mediators to transcription factors. Among them, the core pluripotency transcriptional machinery of ESCs includes Oct4, Sox2, and Nanog, which regulate numerous genes encoding proteins required for pluripotency and self-renewal (1). In coordination with the core pluripotency factors, repressor complexes (e.g. polycomb repressive complexes, and histone deacetylase (HDAC) containing complexes) also play a critical role in the modulation of stemness and differentiation in ESCs (2).

For ESC differentiation research, there are three conventional differentiation protocols commonly used. First, when ESCs are grown using a low-attachment culture dish, ESCs form free-floating spheres, called embryoid bodies (EBs) (3, 4). The second method is direct mESC differentiation, by growing them on extracellular-matrix coated culture dishes as monolayers (5). The last differentiation method is co-culture of ESCs with stromal cells, where direct



contact with neighboring stromal cells induces differentiation (6). These three established differentiation methods have been used to generate a broad range of lineage-specific cells in accordance with on differential supplements.

### *REST/CoREST repressor complex*

REST (RE1 Silencing Transcription factor) is a zinc-finger transcriptional repressor that has a central regulatory role in neural development (7, 8). REST recognizes and binds canonical and non-canonical RE1 motifs (9, 10) and modulates gene expression and long-term silencing (11). The REST complex includes a broad array of binding partners that promote histone modification and chromatin remodeling, recruiting for example scaffold proteins such as CoREST (12, 13). The associations with mSIN3/HDAC, and CoREST/LSD1 at the N- and C- terminal domains of REST, respectively, are essential for REST's effects on gene and epigenetic modulation (14-17). REST is post-transcriptionally modulated via the alteration of its localization, and its ubiquitin-proteasome mediated protein destruction (18, 19).

REST not only controls target gene expression in stem cells, but also contributes to cell identity determination, such as that of embryonic and neural stem/progenitor cells and a number of neuronal phenotypes such as neurite outgrowth and neuronal migration during neural development (20-23). Although the roles of REST in maintaining embryonic stem cell pluripotency remain

controversial (24-27), REST is believed to have functions in maintaining neural stem cells (21), and REST down-regulation leads to the differentiation of embryonic or neural stem cells towards neural lineages (8, 20, 28). Despite the knowledge of REST's downstream target modulation in embryonic and neural stem cell differentiation, identification of upstream signals acting on REST/CoREST modulation remains to be further studied (13).

### *Cadherin-Catenin complex*

Cell-cell adhesion helps to maintain the integrity of differentiated tissues. For example, as a major component of the adherens junction, cadherins assist with providing cell-cell adhesion, enhanced tissue-integrity, and the modulation of cell death (29). Among multiple other things, cadherins play a critical role in early vertebrate development and in maintaining stemness of both human and mouse embryonic stem cells (30, 31). For example, the first identified classic type I cadherin, E-cadherin is required for the proper development of mammalian embryos, with the lack of E-cadherin in embryos leading to defective morula compaction and blastocyst malformation (32).

Additionally, E-cadherin enhances the self-renewal and survival of hESCs through decreased caspase activity and increased anti-apoptotic gene Bcl-XL expression (33). The signaling pathways by which E-cadherin contributes to stemness may be complicated. Whereas mESCs maintain stemness in a LIF-dependent manner, E-cadherin knockout (Cdh1<sup>-/-</sup>) mESCs maintain stemness in

a FGF2/Nodal/activin dependent manner, independent of the LIF pathway. Also since the ectopic expression of STAT3 in the Cdh1<sup>-/-</sup> mESCs is sufficient to maintain pluripotency (34), cell-cell adhesion might not be crucial for maintaining pluripotency in this context. However, E-cadherin is believed to be required for stemness in a LIF dependent manner wherein the E-cadherin ectodomain associates with and stabilizes the LIFR and GP130 complex.

Furthermore, E-cadherin expression improves the somatic cell reprogramming of mouse embryonic fibroblasts to form induced pluripotent stem cells (iPSCs) (35, 36). Although E-cadherin appears to be an important component to maintain stemness of ESCs and iPSCs, it is yet unclear whether the stemness is modulated directly by E-cadherin or indirectly via other factors dependent upon E-cadherin mediated cell adhesion.

#### P120-catenin subfamily

At adherence junctions, E-cadherin associates with beta-catenin via the distal region of its cytoplasmic tail, and in parallel, interacts with p120-catenin via the proximal region of its cytoplasmic tail (37, 38). The p120-catenin subfamily (including p120-catenin itself) plays a central role in stabilizing cadherins at adherens junctions, and modulating small-GTPases (e.g. RhoA and Rac) and the actin cytoskeleton (39-41). In addition to its roles at cell-cell junctions and in the cytoplasm, p120-catenin has nuclear roles where it regulates gene expression (42-44). As we reported previously, p120-catenin isoform 1 and beta-catenin

protein levels can be modulated by shared mechanisms, specifically via a well-studied destruction complex that responds to canonical Wnt signals (45, 46). Further, p120-catenin and beta-catenin share certain gene targets, and together modulate them in an additive fashion (44, 47). However, such subsequent target gene modulation mechanisms of p120-catenin are distinct from those of beta-catenin. For example, p120-catenin displaces repressor proteins from gene-control regions to activate their expression, whereas beta-catenin possesses a transactivation domain, and becomes resident at gene promoter sites through indirect interaction with promoter or enhancer regions (48). That is, beta-catenin can associate with DNA-bound transcription factors such as TCF/LEF to activate target genes (49, 50), whereas p120 leads to gene activation by displacing the Kaiso repressor from its consensus sites in gene regulatory regions (47, 51).

In our current work, we extend our knowledge of the nuclear roles of p120-catenin in a novel direction, and contribute to a better understanding of stem cell biology by revealing that p120 binds to and derepresses the REST/CoREST complex to activate gene targets. As outlined below, our findings suggest that p120 is further involved in regulating differentiation in mESCs, and we indicate a potential mechanism. We find that p120 participates in modulating the balance of stem cell maintenance versus differentiation in mESCs, and examine potential upstream modulators of the p120-catenin/REST/CoREST pathway. In mESCs, our results suggest that this pathway is coupled to changes in the structure of the cadherin-catenin complex at cell-cell junctions, and that it is then dependent on

p120's nuclear modulation of REST/CoREST. Our findings thus contribute in part to an appreciation of the larger decision process that takes place between maintaining a stem cell state or advancing to a more differentiated one.

## **CHAPTER II**

## **RESULTS**

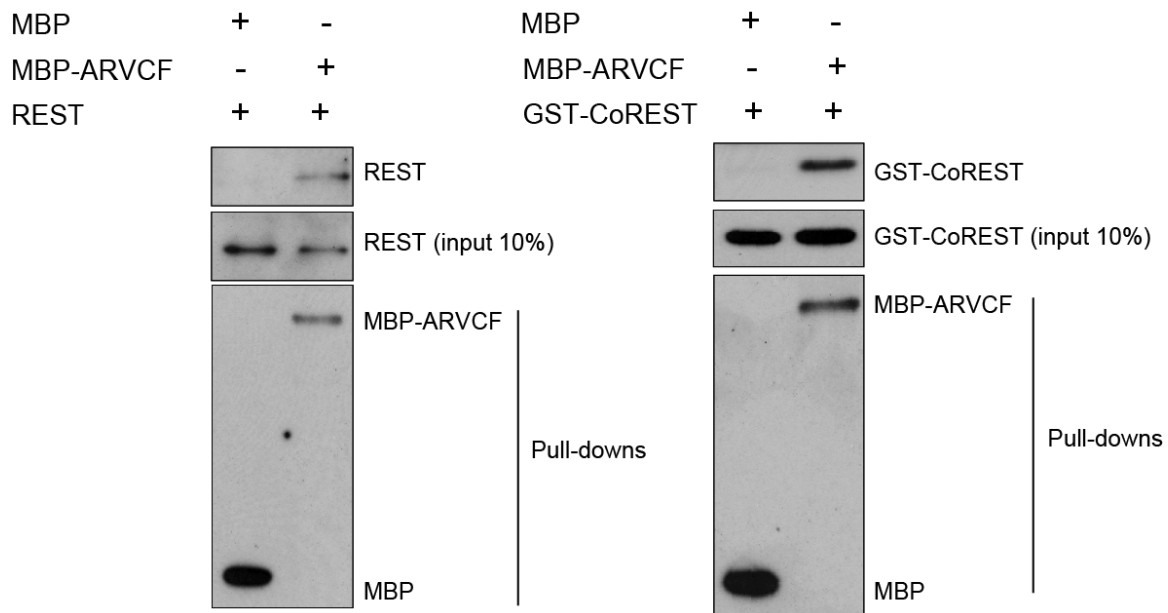
## **Part One: Association of p120-catenin with REST and CoREST**

### *Association of p120-catenin with REST and CoREST in vitro*

In previous work by our research group, we determined that ARVCF-catenin associates with Kazrin (52), which has roles in the cytoplasm and possibly the nucleus as a scaffolding protein (53, 54). To perform yeast two-hybrid screening, a mouse adult brain library was used, from which it was suggested that CoREST may be one of Kazrin's binding partners (Table 1). We further investigated whether ARVCF-catenin, and additional p120-catenin subfamily members including p120-catenin itself associate with CoREST, as well as with CoREST's frequent gene-regulatory partner REST.

To test in vitro whether ARVCF-catenin associates with CoREST and REST, we used maltose binding protein (MBP)-ARVCF, GST-CoREST and REST proteins, as expressed in and purified from bacteria. Compared to MBP alone, MBP-ARVCF was co-precipitated with GST-CoREST (Figure 1). In a manner similar to CoREST:ARVCF association, REST was co-precipitated with MBP-ARVCF, suggesting that ARVCF-catenin might have a shared role with p120-catenin as described below.

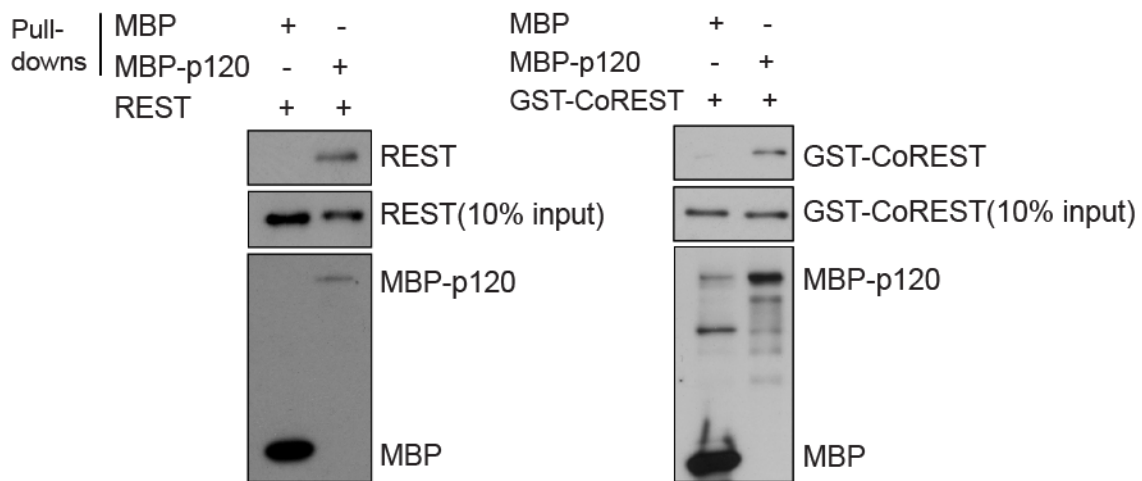
We also performed an in vitro pull-down assay using purified MBP-p120, GST-CoREST and REST to test whether p120 directly associates with REST and CoREST. MBP-p120, but not MBP alone, directly bound to both REST and CoREST (Figure 2).



**Figure 1. ARVCF associates with REST and CoREST in vitro.**

Bacterial produced MBP, MBP-ARVCF, GST-CoREST, and REST proteins were purified using conventional methods. REST was then cleaved from the GST-REST: Glutathione Sepharose 4B bead complex by use of thrombin protease. REST or GST-CoREST were incubated with either MBP or MBP-ARVCF, and MBP or MBP-ARVCF was precipitated using amylose beads. The indicated proteins were detected with anti-MBP, anti-GST, and anti-REST antibodies. While MBP was not co-precipitated, MBP-ARVCF was co-precipitated with REST and GST-CoREST.





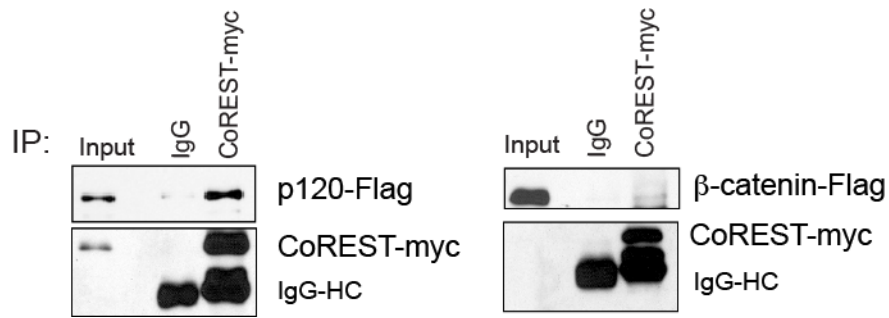
**Figure 2. P120 associates with REST and CoREST in vitro.**

Either purified MBP or MBP-p120 was incubated with GST-CoREST, and REST proteins for direct binding assays, followed by pull-down using amylose magnetic beads. The CoREST and REST proteins were detected by blotting with anti-GST, and anti-REST antibodies, respectively. Anti-MBP antibody was used to detect precipitated MBP and MBP-p120 proteins. Both REST and CoREST proteins were co-precipitated with MBP-p120, whereas MBP alone interacted to an undetectable extent.

### *Association of exogenous p120-catenin and REST/ CoREST in mammalian cells*

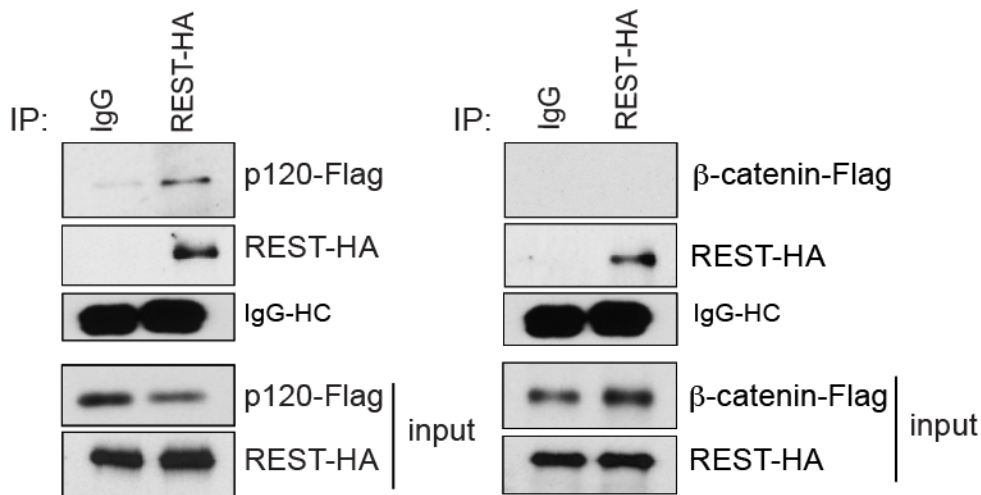
As a scaffolding component of the REST repressor complex, CoREST contributes to transcriptional repression of gene targets (16). To assess the protein interaction between p120-catenin and CoREST, exogenous p120 and CoREST were co-expressed in HeLa cells using a liposome-mediated transfection method. Consistent with the in vitro pull-down assay, as compared to IgG control precipitation, p120 was co-precipitated with CoREST in HeLa cells (Figure 3). Furthermore, we assessed the specificity of its association by testing the interaction of beta-catenin and CoREST, with beta-catenin serving as a good control given that it is structurally similar to p120-catenin (eg. each harboring a central armadillo domain). Although beta-catenin was expressed at higher levels than p120-catenin, its association with CoREST was barely detected relative to that with p120, suggesting specificity in the p120-CoREST interaction. We next tested whether exogenous p120-catenin associates with REST in HeLa cells. As for the interaction tests for p120:CoREST, epitope-tagged REST and p120-catenin or beta-catenin proteins were expressed in HeLa cells using liposome-mediated transfection methods. As determined for p120:CoREST association, we observed that p120-catenin associates with REST, relative to negative control IgG precipitations. Likewise, exogenous beta-catenin was not co-precipitated with REST relative to that of p120-catenin (Figure 4). These findings were further tested in additional cell lines. For example, consistent with the exogenous p120:REST co-precipitation from HeLa cells, specific co-immunoprecipitation of

p120-catenin and REST was observed in HEK293 cells relative to IgG (negative control) precipitations.



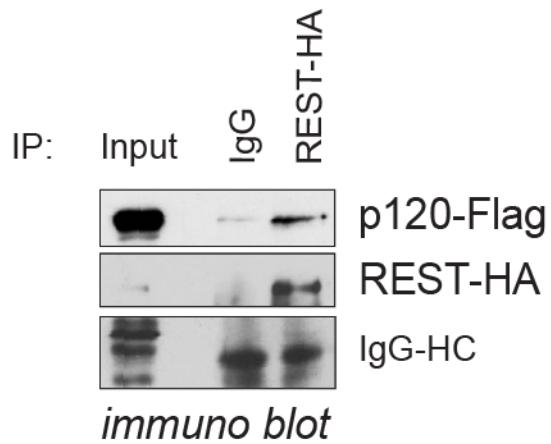
**Figure 3. Exogenous p120-catenin associates with CoREST in HeLa cells, but beta-catenin does not.**

Using liposome-mediated transfection methods, p120-catenin or beta-catenin was co-transfected with CoREST-myc in HeLa cells. Anti-myc antibody was used to precipitate CoREST-myc protein. Immunoblotting employed anti-Flag antibody to test for co-precipitation of p120 versus beta-catenin. Flag-p120 displayed a strong interaction with myc-CoREST relative to the Flag-beta-catenin. Five percent of the lysates used for co-immunoprecipitation were loaded as input.



**Figure 4. Exogenous p120-catenin, but not beta-catenin, associates with REST in HeLa cells.**

Using a liposome-mediated DNA transfection method, REST was co-expressed with p120-catenin or beta-catenin in HeLa cells. 48hrs after transfection, cells were lysed and REST-HA was precipitated using anti-HA antibody, followed by immunoblotting with anti-Flag antibody to detect exogenous p120 or beta-catenin. Exogenous p120 was co-precipitated with REST, while exogenous beta-catenin was not. Ten percent of the lysate volume used for the immunoprecipitations were loaded as input.

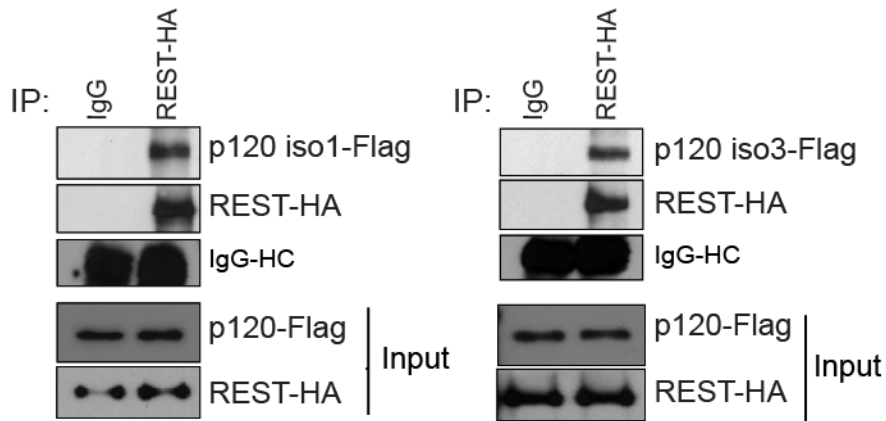


**Figure 5. Exogenous p120-catenin associates with REST in HEK293 cells.**

Using liposome-mediated transfection, p120-flag and REST-HA were transiently co-expressed in HEK293 cells. 48hrs after transfection, cells were lysed and lysates incubated with control IgG or anti-HA antibody to precipitate REST-HA protein. Anti Flag-antibody was used for immunoblotting to detect p120-Flag co-precipitation. Ten percent of lysate volume was loaded as input.

*Association of REST with both isoform 1 and 3 of p120-catenin in vitro*

To further test the association between p120 and REST, we performed binding assays using an in vitro transcription and translation (TnT) system. In a context dependent manner, differential p120 isoforms are generated from distinct translational start sites in the same transcript, or from pre-RNA splicing (55). For instance, while translation of p120 isoform1 starts at the first translation start site, isoform 3 translation initiates at a down-stream translational start site. The in vitro binding assays displayed that both isoform1 and isoform3 bind to REST (Figure 6).



**Figure 6. Both p120 isoform1 and isoform3 associate with REST, as indicated using an in vitro transcription and translation system.**

P120 isoform1-Flag, p120 isoform3-Flag, and REST-HA were prepared using an in vitro transcription and translation (TnT) system. P120 isoforms 1 and 3 proteins were incubated with REST-HA protein and REST-HA was precipitated using anti-HA antibody. Both isoforms were detected using anti-Flag antibody. Relative to an IgG negative control precipitation, both p120 isoforms were co-precipitated with REST.

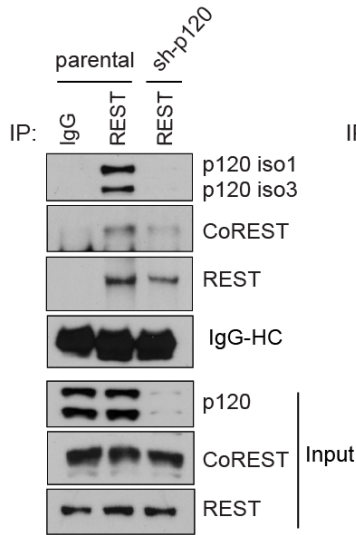


### *Association of endogenous p120-catenin and REST/ CoREST*

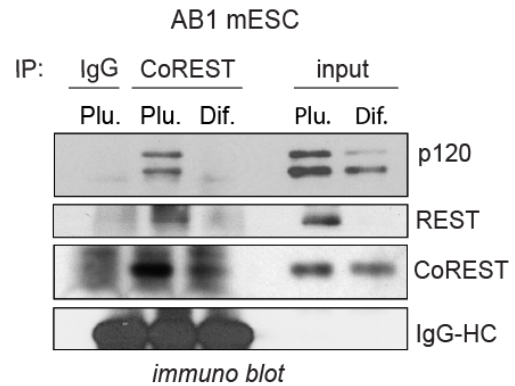
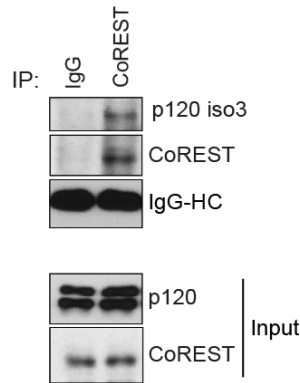
Based on the evidence for the interactions between p120 and REST/CoREST in vitro and protein overexpression systems, we asked whether endogenous p120 associated with REST and CoREST in a number of cell lines including mESCs. Although the REST/CoREST complex has been well studied in mESCs, the role of p120-catenin in mESCs remains unknown. Using conventional co-immunoprecipitation methods, we observed that both endogenous p120-isoform 1 and isoform 3 had a strong interaction with REST, as compared to IgG negative control precipitations (Figure 7A). The p120 co-precipitation was not observed in p120 knockdown mESCs, confirming that endogenous p120 associates with REST in mESCs (Figure 7A). Also endogenous p120 interacted with endogenous CoREST in mESCs (Figure 7A). Although both endogenous p120 isoform 1 and isoform 3 associated with CoREST in mESCs (Figure 7B), the p120 isoform 3 association with CoREST was more consistently observed (Figure 7A).

To assess the endogenous interactions between p120 and REST/CoREST in an additional cell line, HEK293 cells were used. We observed an endogenous p120 and REST/CoREST association in HEK293 cells (Figure 8), consistent with the endogenous interactions observed in mESCs. Interestingly, whereas REST more strongly associated with p120 isoform 1 than isoform 1 [?], CoREST interacted with both p120 isoform 1 and 3 in HEK293 cells, suggesting that different cells may have differential p120:REST/CoREST interaction patterns. Although it remains unknown, the context dependent association patterns may relate to

distinctive roles of p120 isoforms according to context. In all cases, our data suggest that p120-catenin binds to both REST and CoREST in various cell types, including mESCs.



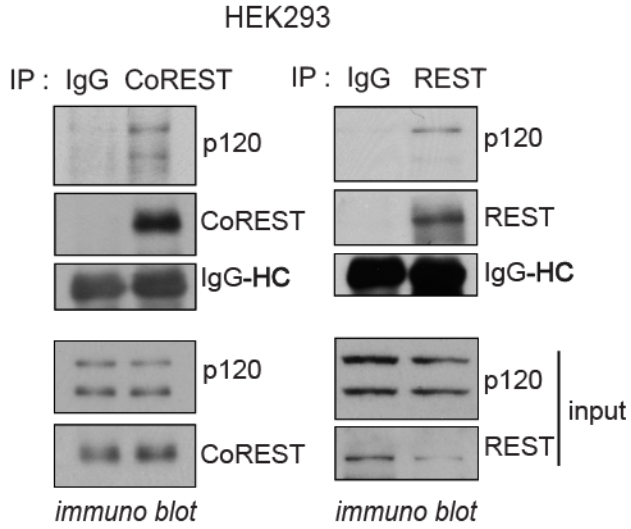
**Figure 7A**



**Figure 7B**

### Figure 7. Endogenous p120-catenin associates with REST and CoREST in mESCs.

Nuclear fractions were obtained from AB1 mESCs. To assess endogenous p120 and REST/CoREST association, endogenous REST and CoREST were precipitated with anti-REST and anti-CoREST antibodies, respectively. The indicated endogenous proteins were detected by blotting with anti-p120, anti-CoREST and anti-REST antibodies (Figure 7A). For Figure 7B, both pluripotent and differentiating AB1 stem cells were used to isolate the nuclear fractions. To promote stem cell differentiation, embryoid body formation was followed by retinoic acid treatment for 2days prior to harvest. Endogenous CoREST was precipitated using anti-CoREST antibody (Figure 7B) (Note: Plu.=pluripotent ESCs; Dif.= differentiating mESCs).



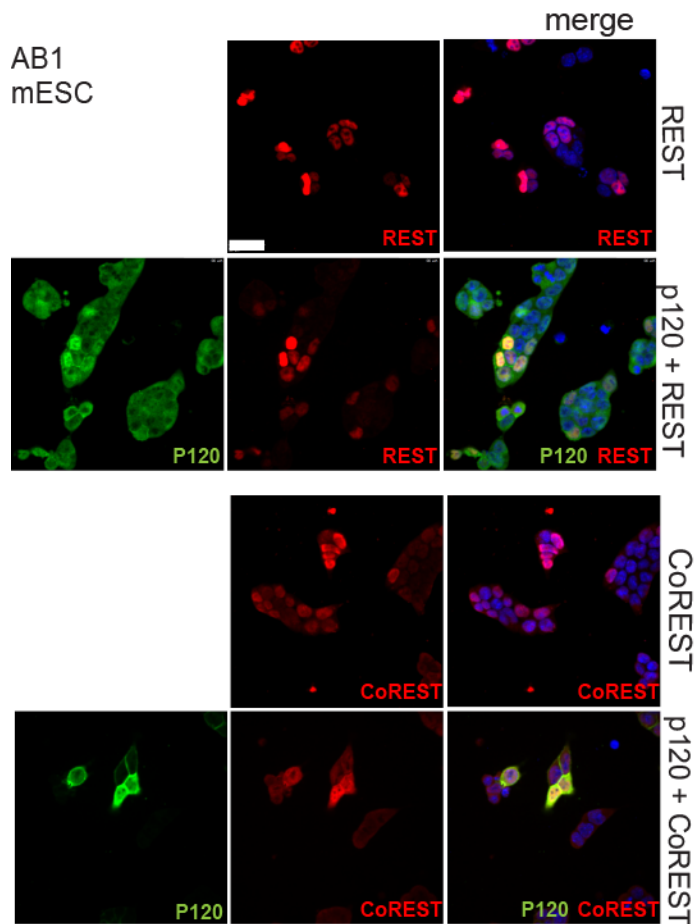
**Figure 8. Endogenous p120-catenin associates with REST and CoREST in HEK293 cells.**

To assess endogenous protein interactions, nuclear fractions were obtained from HEK293 cells, and endogenous CoREST and REST proteins were precipitated using anti-CoREST and anti-REST antibodies. Anti-p120 antibody was used to detect endogenous p120.

*P120 co-localizes with REST and CoREST in mESCs.*

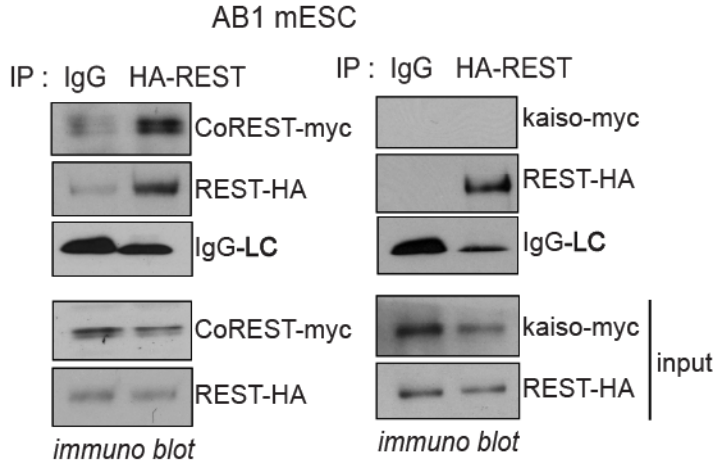
To investigate the effect of p120-catenin expression on the localization of REST/CoREST in mESCs, we performed immunostaining using AB1 cells expressing exogenous p120 and/or REST or CoREST (Figure 9). When REST alone was expressed in mESCs it was predominantly evident in the nucleus, which is consistent with the previous reports. When p120-catenin and REST were co-expressed in mESCs, p120 partially co-localized (at the limited resolution of the immuno-staining method) with REST in the nucleus. We likewise observed strong exogenous CoREST localization to the nucleus. However, its localization was not limited to the nucleus, as immuno-staining indicated some cytoplasmic presence in mESCs. Upon expression of p120 with CoREST, p120 co-localized with CoREST in both the cytoplasmic and nuclear compartments, and the relative fraction of cytoplasmic CoREST became increased. This suggested that p120-catenin might modulate CoREST's cytoplasmic-nuclear distribution, conceivably via transport or sequestration. In contrast, co-localization of p120 with REST in the cytoplasm was not detected, possibly because of rapid ubiquitin-proteasome mediated degradation of REST in the cytoplasmic space (19).

Since p120-catenin associates with Kaiso (44), and Kaiso can bind to other proteins promoting gene repression such as N-CoR (56), we tested whether Kaiso might be part of the REST/CoREST repressor complex. Relative to the REST:CoREST interaction in AB1 cells, Kaiso did not associate with REST (Figure 10), suggesting that Kaiso is not a component of the REST complex.



**Figure 9. p120-catenin co-localizes with REST and CoREST in mESCs.**

Using lipofectamine 2000, p120-catenin and REST or CoREST cDNA were transfected into AB1 cells. 24hrs after transfection, the cells were fixed with 4% formaldehyde for 10min, and incubated with PBS made 0.3% in TritonX100 for permeabilization. Anti-HA and anti-myc antibodies were used for exogenous protein immuno-detection under 3i Confocal microscopy (Zeiss). DAPI was used for counter-staining. Scale bar represents 30 $\mu$ m.



**Figure 10. Kaiso is not part of the REST complex in mESCs.**

Using lipofectamine 2000, REST, CoREST or Kaiso cDNAs were transfected into AB1 cells. 48hrs after transfection, cells were harvested. For co-immunoprecipitation assays, whole cell lysates were used. Exogenous REST was precipitated using anti-HA-probe antibody. CoREST and Kaiso were detected using anti-myc antibody. Five percent of lysate volumes used for co-immunoprecipitation were loaded as input.

**Part Two: The zinc finger/DNA-binding domain of REST, and the region between CoREST's two SANT domains, associate with the Armadillo domain of p120-catenin.**

*REST binding domain mapping with p120-catenin*

To assist in discerning roles of the p120:REST association, we conducted protein interaction domain mapping of the p120:REST association using an in vitro transcription and translation system. REST contains protein and DNA interaction regions. SIN3B associates with the amino-terminal region of REST, and CoREST interacts with the carboxyl-terminal region of REST. The zinc finger domain of REST associates with sequence-specific RE1 consensus DNA sites of REST gene targets (57). Our co-immunoprecipitation data indicates that the zinc finger domain of REST is required for selective binding to p120, relative to the other REST fragments (Figure 11). Thus, REST binding domain mapping implied that p120-catenin may play a role in the modulation of REST gene targets.

*CoREST interaction domain mapping with p120-catenin*

CoREST contains two highly conserved SANT (Swi3, Ada2, N-Cor, and TFIIIB) domains that are required for protein:protein interactions. For example, the first SANT domain of CoREST associates with REST and HDAC1 (58), whereas the second SANT domain binds Brg1 associated factor 57 (BAF57) (59). To determine CoREST interaction mapping of p120, based on this information, we designed seven fragments of CoREST to perform in vitro binding assays using



full-length p120-catenin. Surprisingly, neither the first or second SANT domain associated with p120. Instead, p120 associated with the region between the two SANT domains (Figures 12). Although additional CoREST interaction domain mapping is required to determine a more precise binding region, this finding suggests that p120 might not compete for REST association with CoREST, but that the three proteins may form a trimetric complex (see below).

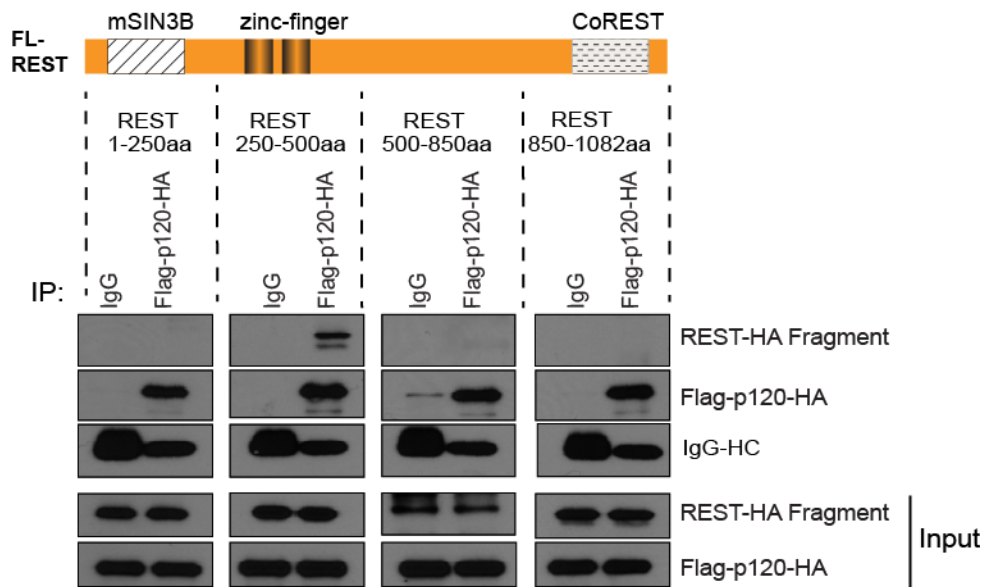
#### *P120-catenin interaction domain mapping with CoREST and REST*

Next, we tested which p120-catenin region is required for REST and CoREST association. P120 contains approximately ten Armadillo repeats in series, and this larger Armadillo domain plays important roles in associations with binding partners such as Kaiso and E-cadherin (60, 61). Based around the Armadillo domain p120, we designed amino-terminal, carboxyl-terminal and Armadillo domain fragments. Using an in vitro transcription and translation system, the indicated proteins were generated, and conventional in vitro co-immunoprecipitation assays were performed. Relative to carboxyl-terminal fragments, the Armadillo domain had a selective interaction with CoREST (Figure 13). Also the Armadillo domain strongly associated with REST, compared to amino-terminal and carboxyl-terminal fragments of p120 (Figure 14). The carboxyl-terminal fragment of p120 displayed a very weak association with REST, perhaps due to the approximately 1.5 Armadillo repeats retained in the carboxyl-terminal fragment construct (Figure 14). However, compared to the Armadillo-domain:REST association, the carboxyl-terminal fragment of p120-

catenin displayed a considerably weaker interaction with REST. Our findings suggest that p120 might regulate REST/CoREST gene targets via an impact on both the REST:CoREST protein:protein interaction and of REST with RE1 DNA consensus sites.

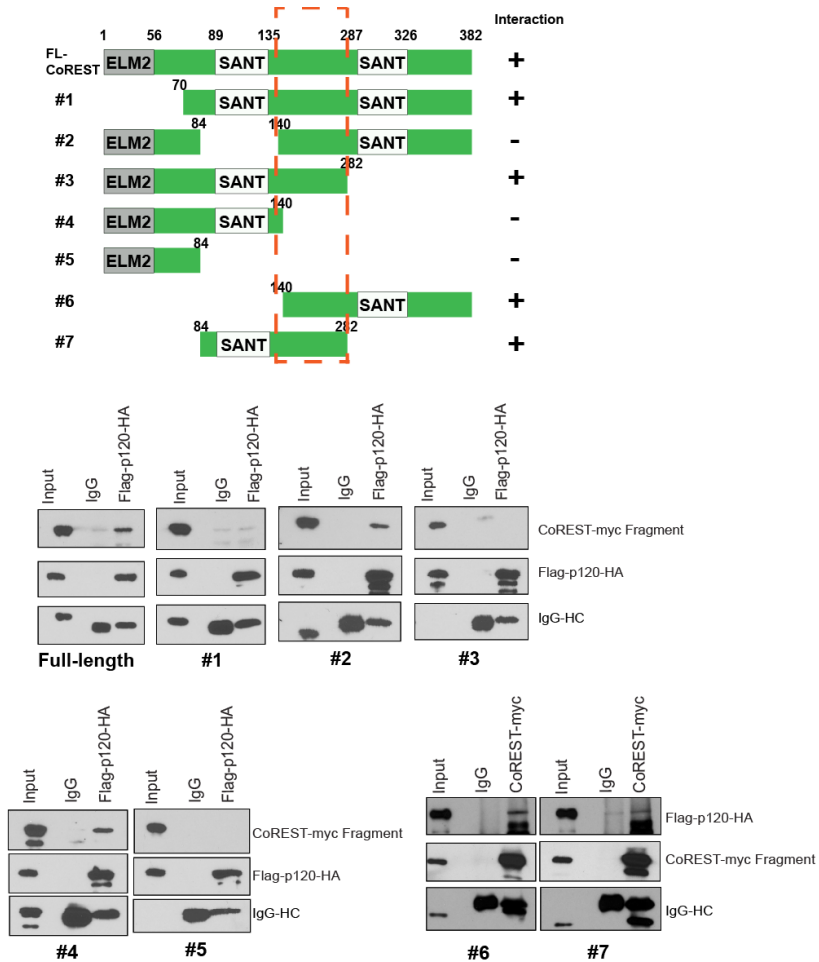
*P120-catenin can associate simultaneously with REST and CoREST.*

Although we observed a direct interaction between p120 and REST and CoREST in vitro (Figure 2), as well as in mammalian cells (Figure 3-8), it remains unclear whether p120 simultaneously forms a complex with REST and CoREST or whether p120 separately associates with REST and CoREST. Using a REST carboxyl-terminal deletion mutant that can no longer bind CoREST (16), we conducted in vitro binding assays to address this question. We confirmed that the REST deletion mutant did not bind to CoREST, in contrast to full-length REST (Figure 15). In the presence of p120, both full-length REST and the REST deletion mutant successfully co-precipitated with CoREST. This suggested that p120 can associate with REST and CoREST simultaneously as well as separately.



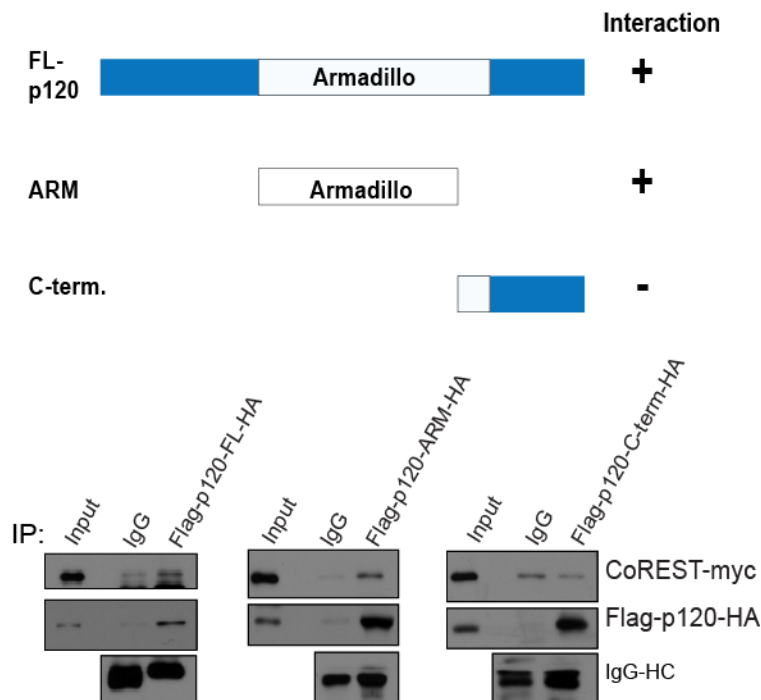
**Figure 11. The zinc finger region of REST associates with p120.**

Using an in vitro transcription and translation system, p120 and the REST fragments were prepared. The REST fragments and p120 were incubated in PBS containing 0.3% TritonX100, and p120 was precipitated with anti-Flag antibody. Both REST fragments that interacted and full-length p120 itself were detected via immuno-blotting using anti-HA antibody.



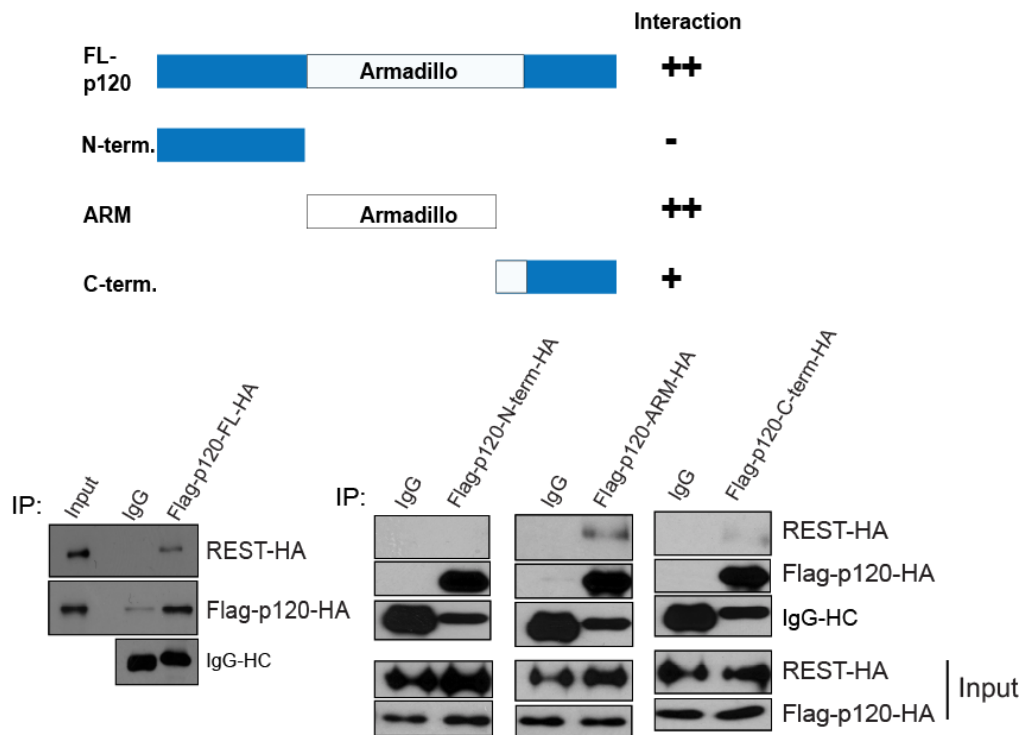
**Figure 12. The region between CoREST's two SANT domains associates with p120.**

The indicated proteins were generated using an in vitro transcription and translation system. CoREST fragments and p120 were incubated in PBS including 0.3% Triton and the indicated proteins were precipitated with anti-Flag or anti-myc antibodies. A dashed red rectangle indicates the CoREST region binding to p120.



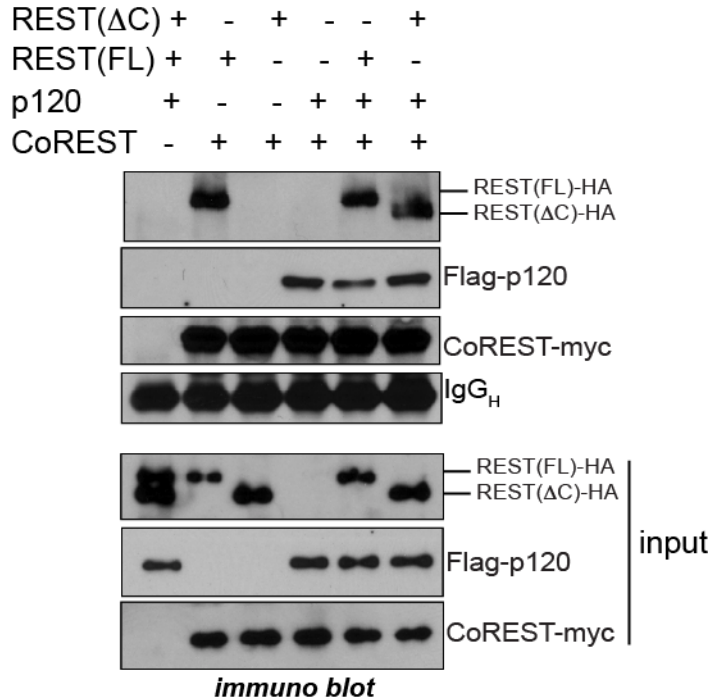
**Figure 13. The Armadillo repeat (ARM) domain of p120 associates with CoREST.**

The indicated proteins were generated using an in vitro transcription and translation system. Anti-Flag antibody was used to precipitate the p120 fragments, and anti-myc antibody was used to detect co-precipitated CoREST. Note. The carboxyl-terminal fragment harbors Armadillo repeats 8.5 and 9.



**Figure 14. The Armadillo repeat (ARM) domain of p120-catenin associates with REST.**

Using an in vitro transcription and translation system, the indicated proteins were generated. Anti-Flag antibody was used to precipitate the p120 fragments, and anti-HA antibody was used to detect co-precipitated REST. Note. The carboxyl-terminal fragment harbors Armadillo repeats 8.5 and 9.



**Figure 15. P120-catenin can simultaneously associate with REST and CoREST.**

Using an in vitro transcription and translation system, REST (full-length and a carboxyl-terminal deletion mutant  $\Delta$ C that encompasses amino acids 1-983), as well as CoREST and p120 were generated. The indicated proteins were incubated in PBS containing 0.3% TritonX100, and CoREST was precipitated using anti-myc antibody. Anti-Flag and anti-HA antibodies were used to detect co-precipitated proteins. Whereas the REST mutant  $\Delta$ C did not associate with CoREST as expected when put together in the absence of p120, REST  $\Delta$ C did co-precipitate with CoREST when p120-catenin was further present.

### **Part Three: P120-catenin modulates REST occupancy at RE1 consensus sites at cis-regulatory regions of target genes.**

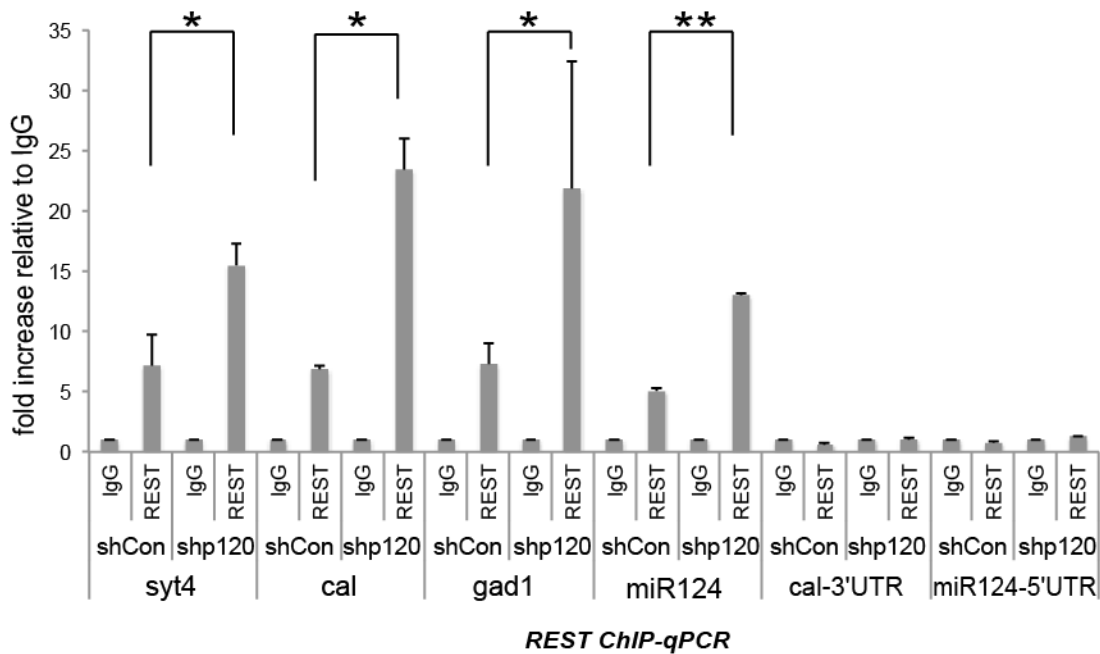
The REST/CoREST repressor complex prevents precocious gene expression that would lead to premature differentiation in neural stem/progenitor cells and embryonic stem cells. REST binds to RE1 sites of gene targets through its zinc-finger region and recruits binding partners involved in gene repression and chromatin remodeling (e.g. CoREST and SIN3B). However, when the zinc finger region is expressed alone, it behaves as a dominant-negative by displacing endogenous REST from RE1 sites and activating expression of REST gene targets in PC12 and L6 cells (7). Whereas some REST gene targets do not possess canonical RE1 consensus sites in the regulatory regions, the RE1 binding site is important for REST's modulation of many of its gene targets (7, 10).

To investigate whether p120-catenin affects REST occupancy at the RE1 sites of its gene targets, we performed ChIP-qPCR using normal versus p120-depleted or p120-overexpressing mESCs. As noted below, in keeping with p120's association with the zinc-finger region of REST, p120 expression reduced REST association with the tested RE1 sites, whereas conversely, p120 depletion enhanced such association.



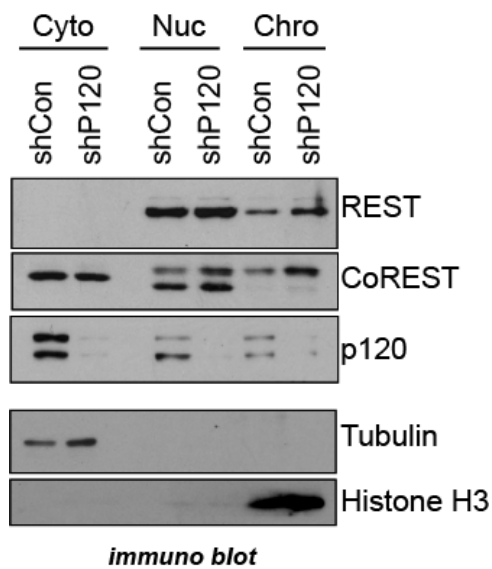
*Molecular mechanism of p120 modulation of REST/CoREST: the impact of p120 depletion on REST occupancy at RE1 consensus sequences*

To determine whether p120 has an effect on REST occupancy at established RE1 sites in known gene targets (e.g., the type II voltage-dependent sodium channel), we conducted ChIP-qPCR using normal and p120 depleted mESCs. We observed that p120 depletion led to REST association with the RE1 sites of four accepted REST targets (synaptotagmin4, calbindin, gad1, and miR-124), with 2-to-4 fold changes observed relative to negative control IgG precipitations (Figure 16). A 3' UTR region of calbindin and a 5'UTR region of miR-124 were probed as negative controls given that they do not contain RE1 consensus sites. Next, we tested the subcellular localization of REST and CoREST in p120-depleted mESCs. Compared to fractions isolated from control knockdown cells, the protein levels of REST and CoREST in chromatin and nucleoplasmic fractions increased upon p120 depletion (Figure 17). While we don't know the basis at present, the cytoplasmic, nucleoplasmic, and chromatin fractions of CoREST exhibited slight differences in their SDS-PAGE mobility. Together, both our ChIP and immunoblotting experiments suggest that p120-catenin and REST may be functionally related to REST/CoREST gene target regulation in mESCs.



**Figure 16. REST occupancy at RE1 regions is increased upon p120 depletion.**

Using an shRNA mediated knockdown approach, control or p120-catenin depletions were undertaken in AB1 mESCs and ChIP-qPCR was performed. To precipitate REST-DNA cross-linked complexes, 2ug of anti-REST or control (rabbit IgG) antibodies were used. As negative control, the UTR regions (lacking RE1 consensus sites) of calbindin and miR124 were used. Data are normalized to the IgG input value and presented as mean values. Error bars represent SD. (one asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ )



**Figure 17. REST and CoREST protein levels were increased upon p120-catenin depletion.**

The indicated fractions were obtained from control and p120-catenin depleted AB1 cells using conventional subcellular fractionation methods. Anti-REST, anti-CoREST and anti-p120 antibodies were used for immunoblotting, and anti-alpha-tubulin and anti-histone H3 were used as a cytoplasmic- and chromatin-fraction marker, respectively.

*Molecular mechanism of p120 modulating REST/CoREST: the impact of p120-catenin overexpression on REST occupancy at RE1 consensus sequences*

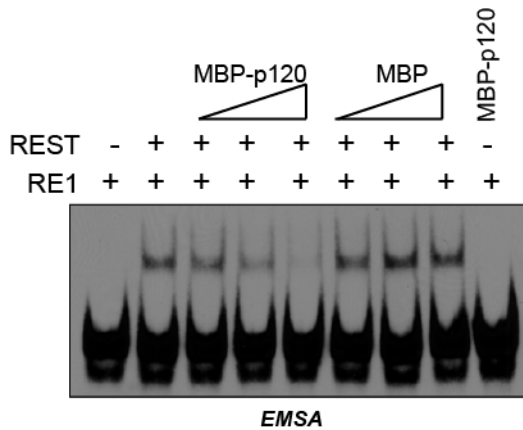
To continue to investigate whether p120 modulate REST occupancy at RE1 sites in known gene targets, we conducted ChIP-qPCR using control versus mESCs expressing exogenous p120. We expected that p120 expression would reduce REST binding to RE1 regions, as opposed to increased REST binding to RE1 sites following p120 depletion (see above).

First, using electrophoretic gel shift assay (EMSA), we tested whether p120 directly affects REST:RE1 DNA complex formation in vitro. We observed that MBP-p120 decreased REST binding to the RE1-containing oligo in a dose dependent manner, whereas MBP alone did not alter REST:RE1 complex formation (Figure 18A). Because the incubation of REST with MBP-p120 or MBP was followed by protein:DNA(RE1) complex formation, these results were consistent with the possibility that p120 prevents REST from binding to RE1-containing DNA by competitively occupying the zinc-finger region of REST. To test whether p120 can also displace REST that is pre-bound to RE1 sites, REST:RE1 complex formation was conducted prior to p120-catenin addition. Interestingly, MBP-p120 displaced REST bound to the RE1-DNA site in a dose dependent manner, with little to no effect of MBP alone on REST displacement. Together, our EMSA findings suggest that p120-catenin can negatively modulate REST:DNA (RE1) complex formation both by preventing REST:RE1 interaction or displacing REST that is pre-bound to RE1-DNA sites.

We next conducted REST ChIP-qPCR using mESCs expressing exogenous full-length p120-catenin. As expected, REST occupancy at established RE1 sites was decreased upon exogenous p120 expression (Figure 19). Although the REST occupancy at the RE1 region of miR-124 was significantly reduced on p120 expression, REST occupancy was only partially decreased at the promoters of calbindin and synaptotagmin4. In parallel, we tested whether a previously characterized NLS mutant of p120 has an effect on REST occupancy at endogenous RE1 sites (51). We confirmed that the p120 NLS mutant largely localized to the cytoplasm (data not shown). Compared to wild-type p120 expression, the NLS mutant did not display a consistent decrease of REST:DNA binding, suggesting that p120 translocation into the nucleus might precede displacement of REST from RE1 regions. Unexpectedly, the p120 NLS mutant increased the endogenous binding of REST to the RE1 site of calbindin. Although we do not understand the mechanism, it may be that the p120 NLS mutant sequestered an unknown factor to the cytoplasm, leading to enhanced REST association with certain gene targets (calbindin) in a context dependent manner.

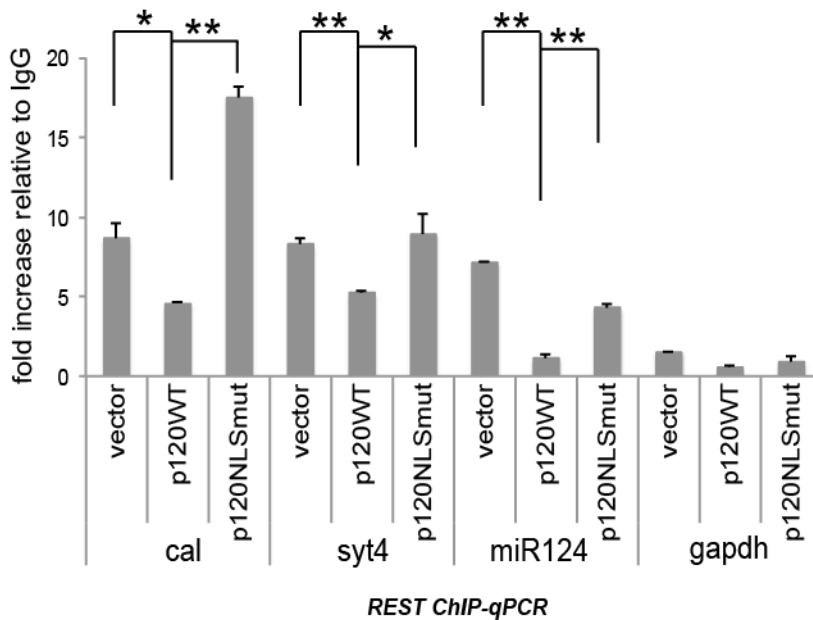
Last, we tested whether the expression of p120's Armadillo domain has an effect on REST:RE1 DNA occupancy, because our prior interaction-domain mapping indicated that the Armadillo domain of p120 interacts with both REST and CoREST. We performed REST ChIP-qPCR using AB1 mESCs that exogenously express defined fragments of p120-catenin. Compared to p120's amino-terminal region and vector control, expression of the Armadillo domain significantly

decreased REST occupancy at the RE1 sites of *gad1*, *calbindin* and *synaptotagmin4*. As expected, expression of p120's Armadillo domain did not have an impact upon REST levels at the 3' UTR of *synaptotagmin4*, as it does not possess an RE1 consensus site (Figure 20). We also observed that the carboxyl-terminal fragment of p120-catenin (containing a small portion of the Armadillo domain), led to a partial decrease of REST:RE1 association, perhaps consistent with its weak association with REST (Figure 14). Although it should be further investigated, the p120 carboxyl-fragment contains half of Armadillo-repeat 8 as well as Armadillo repeat 9, this inclusion may be responsible for the effects observed. We tested that the expression level of each p120 fragment was roughly equivalent (data not shown), and we used AB1 cells expressing the p120 fragments at similar levels for the ChIP-qPCR. Taken together, these findings suggest that p120-catenin modulates REST binding to DNA (RE1) gene-control regions through inhibitory interactions with REST and/ or CoREST. In this manner and as examined below, our findings imply that p120-catenin is likely to modulate (de-repress) REST/ CoREST gene targets.



**Figure 18. P120-catenin decreases REST binding to RE1 region in vitro.**

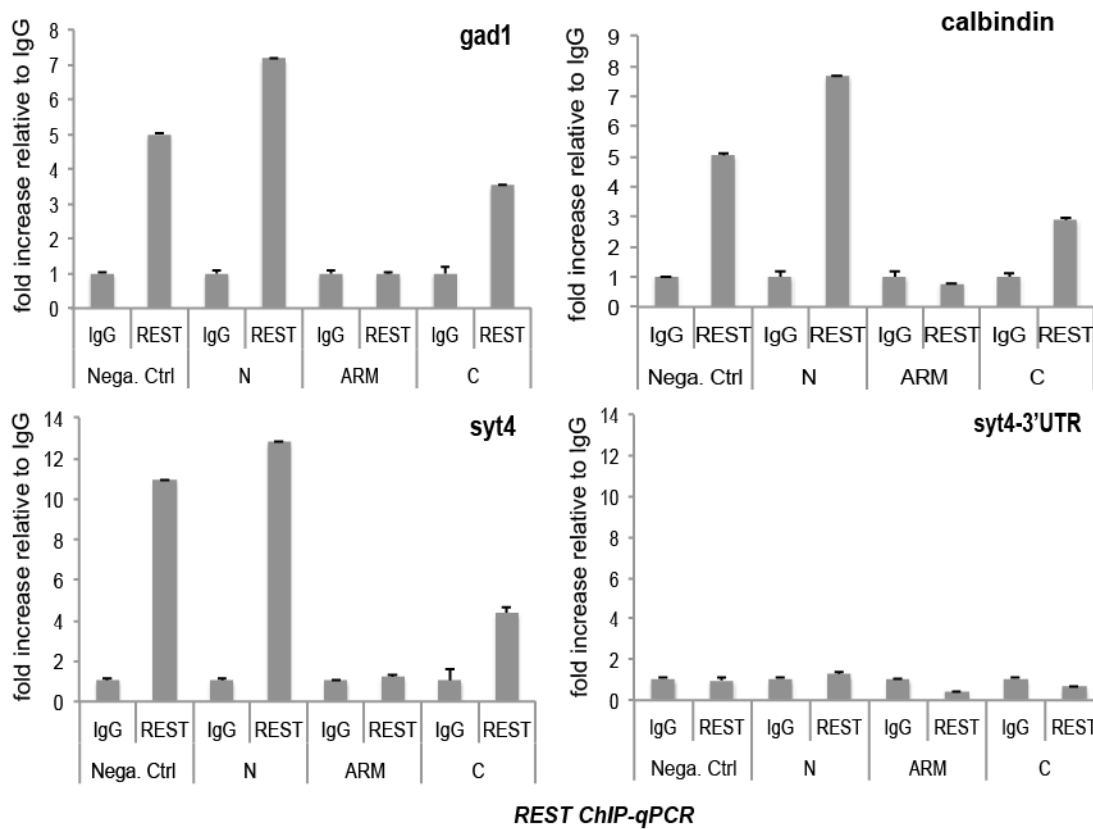
The indicated REST, MBP-p120, and MBP proteins were purified from bacteria, and incubated with a biotin-labeled probe harboring a single RE1 site. The REST:RE1 DNA complex was made in two different ways. For Figure 18A, REST:RE1 probe complex formation preceded incubation with MBP-p120 or MBP alone. For Figure 18B, the incubation of REST with MBP-p120 or MBP alone was followed by REST:RE1 complex formation. The REST:RE1 probe complex was detected using chemiluminescent EMSA (see Materials and Methods).



**Figure 19. P120-catenin expression partially decreases REST occupancy at RE1 regions, but the p120 NLS mutant had little effect.**

The indicated protein cDNA constructs were transfected into AB1 mESCs and 48hrs post transfection, cells were fixed with 1% formaldehyde and harvested for ChIP-qPCR. Anti-REST and control rabbit IgG antibodies were used to pull down REST:DNA complexes. The RE1 regions of three known REST/CoREST gene targets (calbindin, synaptotagmin4 and miR-124) were probed. Gapdh was tested as a negative control. The bars indicate the fold increase compared to the IgG value. Data are normalized to the IgG input value and presented as the mean. Error bars represent SD. (one asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ )





**Figure 20. P120's Armadillo domain decreases REST occupancy at RE1 containing DNA regions of REST gene targets.**

ChIP-qPCR was performed of AB1 mESCs expressing the indicated fragments of p120-catenin: N-terminal (N); Armadillo domain (ARM); or C-terminal (C). Anti-REST antibodies were used to precipitate REST:DNA complexes, while anti-rabbit IgG antibodies served as a negative control. The 3'UTR region of Syt4 was used as an internal-transcript negative control. Data are normalized to the IgG input value and presented as mean values. Error bars represent SD (one asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ )

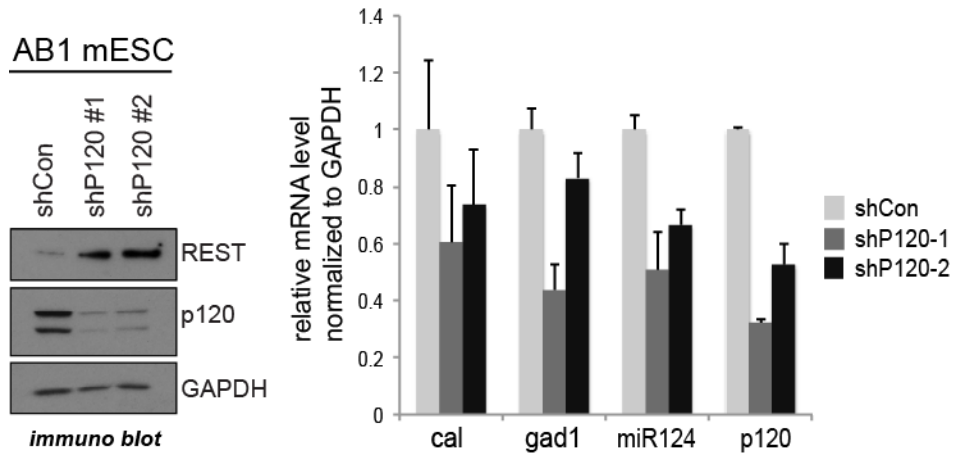
**Part Four: P120-catenin modulates REST/CoREST gene targets in mESCs, in mammalian cell lines, and in *Xenopus laevis* embryos.**

The protein interaction domain mapping and REST ChIP-qPCR indicated that p120-catenin's interaction with the zinc finger/DNA-binding region of REST has an effect on REST binding to gene-control regions of target genes. Next, we tested whether p120 affects REST/CoREST gene target expression by manipulating the expression level of 120.

*The effect of p120 depletion on REST gene target transcription in mESCs*

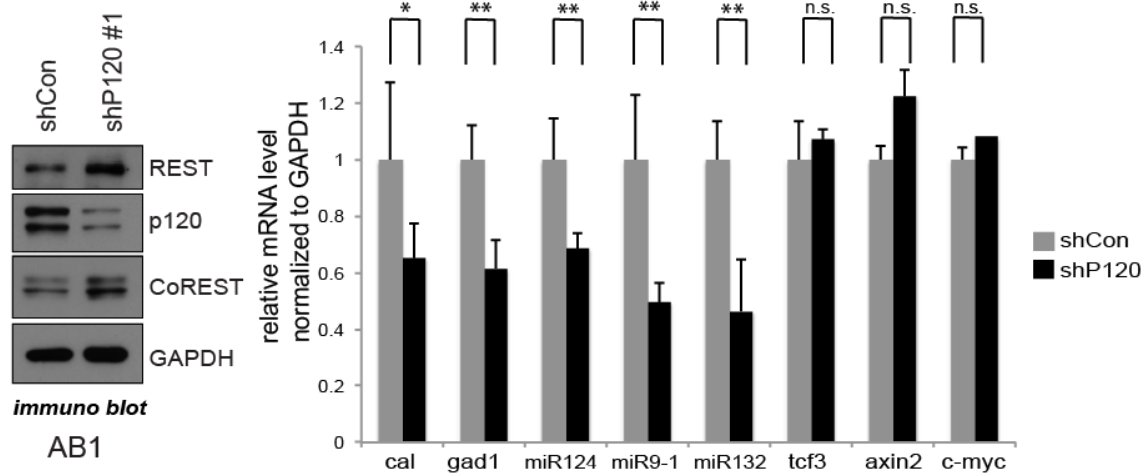
First, we tested whether p120 depletion affects the protein levels of REST in mESCs. Interestingly, REST protein increased upon use of either of two independent p120-directed shRNAs (Figure 21), suggesting that p120 depletion may decrease transcription of REST gene targets. As expected, the transcript levels of most tested REST/ CoREST gene targets (calbindin, gad1, miR-124, miR-9-1, and miR-132) were reduced following p120 depletion (Figure 21&22). However, the transcript level of tcf3 did not appear to be affected by p120 depletion (Figure 22), suggesting that p120 depletion has some selectivity in REST gene target modulation. Also, but as expected, p120 knockdown did not lead to repression of non-REST targets such as axin2 and c-myc (Figure 22). The fact that p120-catenin depletion exhibited a partial as opposed to graphic effects on REST targets may be due to reasons including incomplete p120 knockdown, the involvement of other cis-acting transcription factors, or the

regulation of REST/CoREST targets by other p120-catenin subfamily members such as ARVCF-catenin or delta-catenin. As we observed that ARVCF directly interacts with REST and CoREST in vitro (Figure 1), we assessed whether the depletion of both ARVCF and p120 in mESCs had an additive effect on the transcriptional repression of REST targets. Although the co-depletion of ARVCF and p120 was successfully conducted, the co-depletion did not appear to result in greater effects than observed upon p120-depletion alone (Figure 23). Therefore, other (unknown) cis-acting transcriptional regulators, or the involvement of other p120-subfamily members, such as delta-catenin, may account for moderate effect of p120-depletion alone upon REST/CoREST targets.



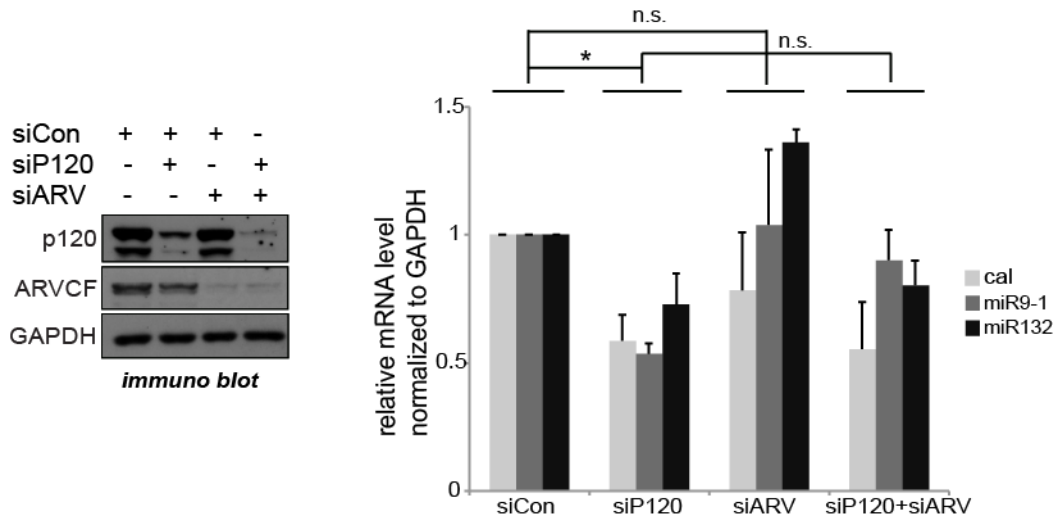
**Figure 21. P120-catenin depletion decreases the transcription of REST/CoREST gene targets.**

Using two independent shRNA constructs, p120 depleted AB1-mESCs were generated, and maintained in selective media (puromycin). Anti-p120 antibody was used to confirm p120 knockdown, and the transcript levels of REST targets were evaluated by real-time PCR, normalized to gapdh. Error bars represent SD.



**Figure 22. P120-catenin depletion (shP120 #1-mediated) decreases transcript levels of REST/CoREST gene targets.**

Using shRNA-mediated RNAi, p120 depleted AB1-mESCs were generated and maintained in selective media (puromycin). Anti-p120, anti-REST, anti-CoREST and anti-GAPDH antibodies were used for immunoblotting. The indicated REST/CoREST gene target transcripts and non-REST/CoREST gene target transcripts were tested by real-time PCR. Gapdh was used as an internal control. Data are presented as mean values. Error bars represent SD (one asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ; n.s., not statistically significant).



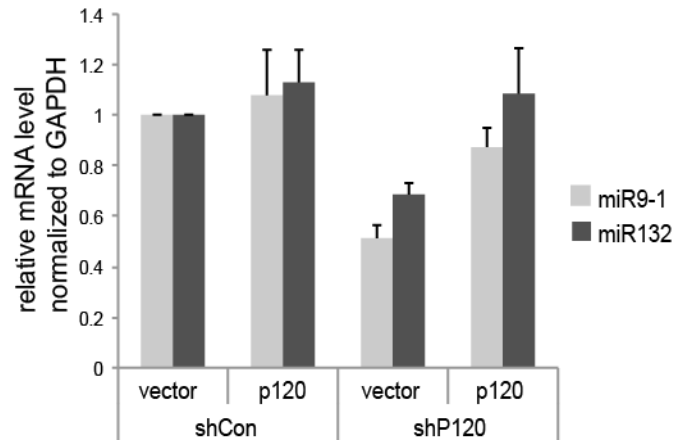
**Figure 23. Co-knockdown of ARVCF and p120 does not increase p120 depletion effects on REST/ CoREST gene targets.**

The indicated siRNAs were transfected into AB1-mESCs. 72hrs after transfection, the cells were harvested for immuno-blotting and real-time PCR. Anti-p120 and anti-ARVCF antibodies were used to confirm the knockdown of p120 and ARVCF, respectively. Gapdh was used as an internal control. Data are presented as mean values, and error bars represent SD (one asterisk,  $P < 0.05$ ; NS, not statistically significant).

To determine whether the increased protein levels of REST and CoREST following p120 depletion contributed to the observed effects upon REST/CoREST gene targets, we performed depletion of REST or CoREST using short interfering RNA (siRNA) in combination with shRNA-mediated p120 knockdown. Prior to this, we tested (positive control) whether p120-depletion effects could be rescued by transfecting non-targetable p120 cDNA (not including UTR region) into p120-depleted mESCs. Indeed, to a notable extent, the expression of non-targetable p120 successfully rescued transcription of miR-9-1 and miR-132 in p120-depleted mESCs (Figure 24).

In addition to rescuing by non-targetable p120 expression, siRNA mediated REST knockdown could significantly but not completely rescue p120 knockdown effects in mESCs (Figure 25).

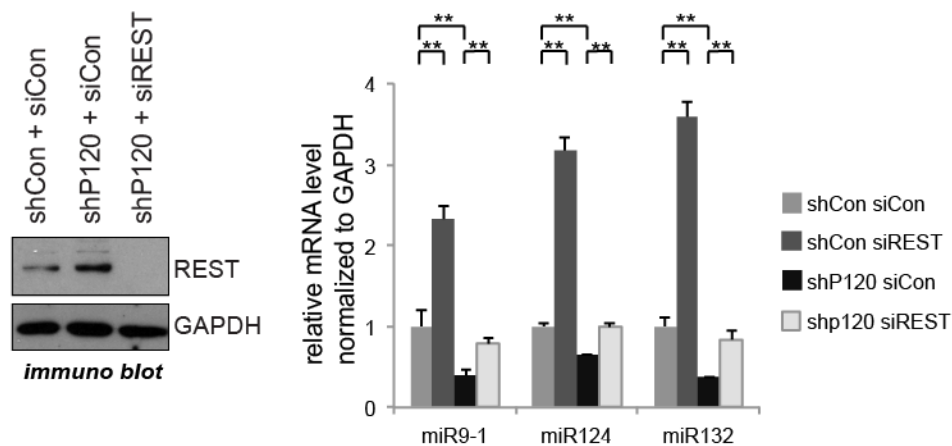
Intriguingly, CoREST knockdown in p120-depleted mESCs increased the transcription of REST/CoREST target genes up to 4-10 fold relative to control conditions (Figure 26). This result may be in part accounted for by previous reports that even independent of REST, CoREST modulates a proportion REST/CoREST gene targets. This is likely to occur via interactions with other transcription factors or via binding to DNA directly (18).



**Figure 24. Non-targetable p120-catenin rescues p120 knockdown effect on REST/CoREST gene targets.**

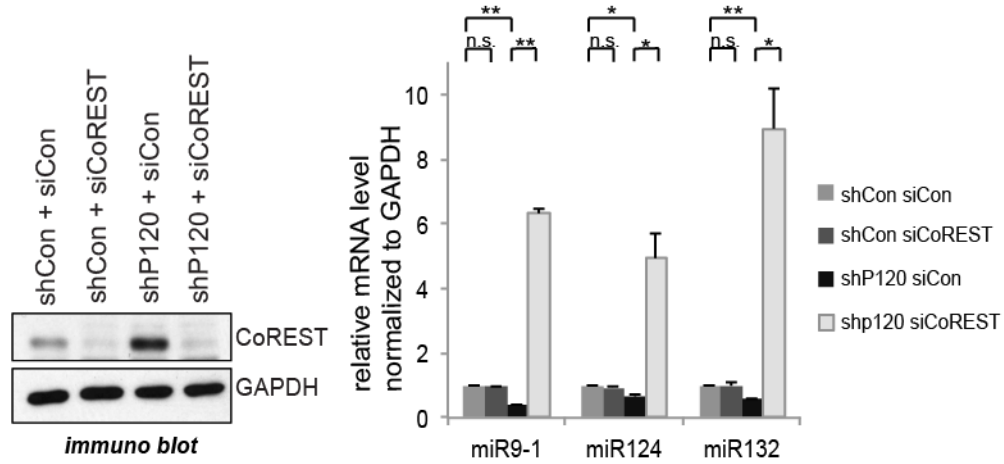
A non-targetable p120 cDNA (lacking UTR region) was transfected into control versus p120 depleted AB1-mESCs. 48hrs post-transfection, RNAs were extracted for qPCR. The indicated REST/CoREST gene targets, miR9-1 and miR132, were tested by real-time PCR. Transcripts were normalized to gapdh. Error bars represent SD.





**Figure 25. REST knockdown partially rescues p120-depletion effects.**

To deplete REST, siRNAs targeting REST were transfected in control and p120-depleted AB1-mESCs. The REST knockdown was confirmed by immunoblotting. Transcription of the indicated REST/CoREST gene targets was tested by real-time PCR. Transcripts were normalized to gapdh. Data are presented as mean values, and error bars represent SEM.



**Figure 26. CoREST knockdown rescues p120-depletion effects.**

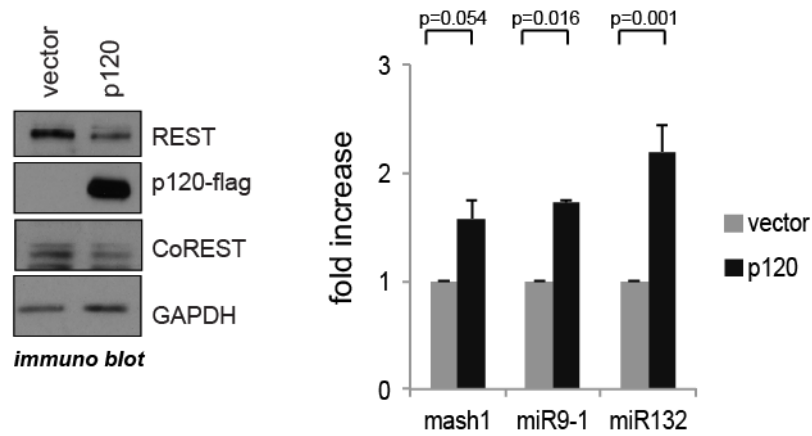
Using siRNA-mediated knockdown, CoREST was co-depleted in control- and p120-depleted AB1-mESCs. CoREST depletion was confirmed by immunoblotting. The indicated REST/CoREST gene target transcription was tested by real-time PCR. All transcripts were normalized to gapdh. Data are presented as mean values, and error bars represent SEM.

*The effect of p120-catenin expression on REST/CoREST gene target transcription in mESCs*

To complement determination of p120-depletion effects on REST/CoREST gene targets, we determined whether p120-catenin expression played a role in REST/CoREST target gene modulation. We observed that REST/CoREST gene target transcription was increased 1.5-to-2 fold upon p120 expression in AB1-mESCs (Figure 27). The p120 expression effect presumably arose from decreased REST and CoREST protein levels via proteasome-mediated degradation (Figure 28), and/ or p120-mediated displacement of REST from RE1 DNA consensus regions (Figure 18&19).

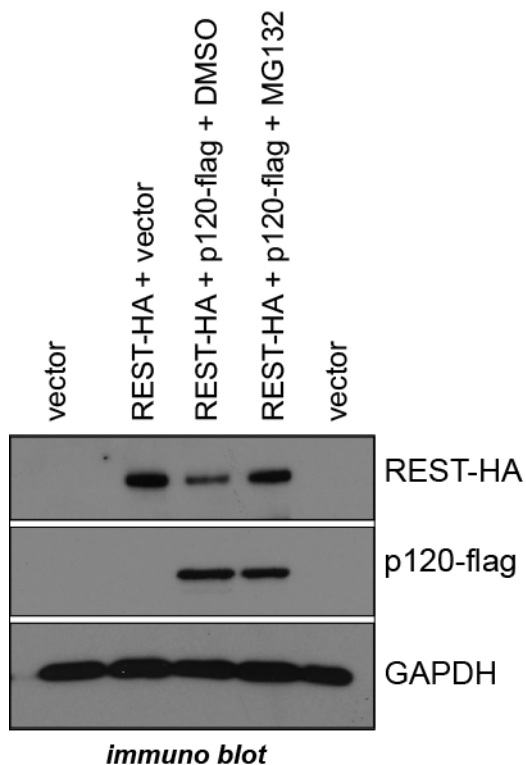
*The effect of p120-catenin on the protein level of REST and CoREST in mESCs*

We observed that the protein level of p120 had an impact on the protein levels of REST and CoREST. For example, p120 depletion reproducibly led to increased REST and CoREST protein levels (Figure 21&22). In a complementary manner, exogenous p120 expression decreased REST and CoREST protein levels (Figure 27). Since differential p120 protein levels did not alter REST and CoREST transcription levels (data not shown), the results suggest that p120 may promote the degradation of REST or CoREST proteins. Indeed, the effect upon REST protein levels following p120 expression was rescued by treatment of cells with the proteasome inhibitor (MG-132; Figure 28), suggesting that p120 may lead to REST protein degradation through proteasome-mediated mechanisms.



**Figure 27. P120-catenin expression promotes REST/CoREST gene target transcription.**

48hrs post-transfection of p120-catenin, AB1-mESCs were harvested for immunoblotting and real-time PCR. The indicated antibodies were used for immunoblotting and GAPDH used as an internal control. The REST/CoREST gene transcripts were tested by real-time PCR and normalized to gapdh. Error bars represent SEM.

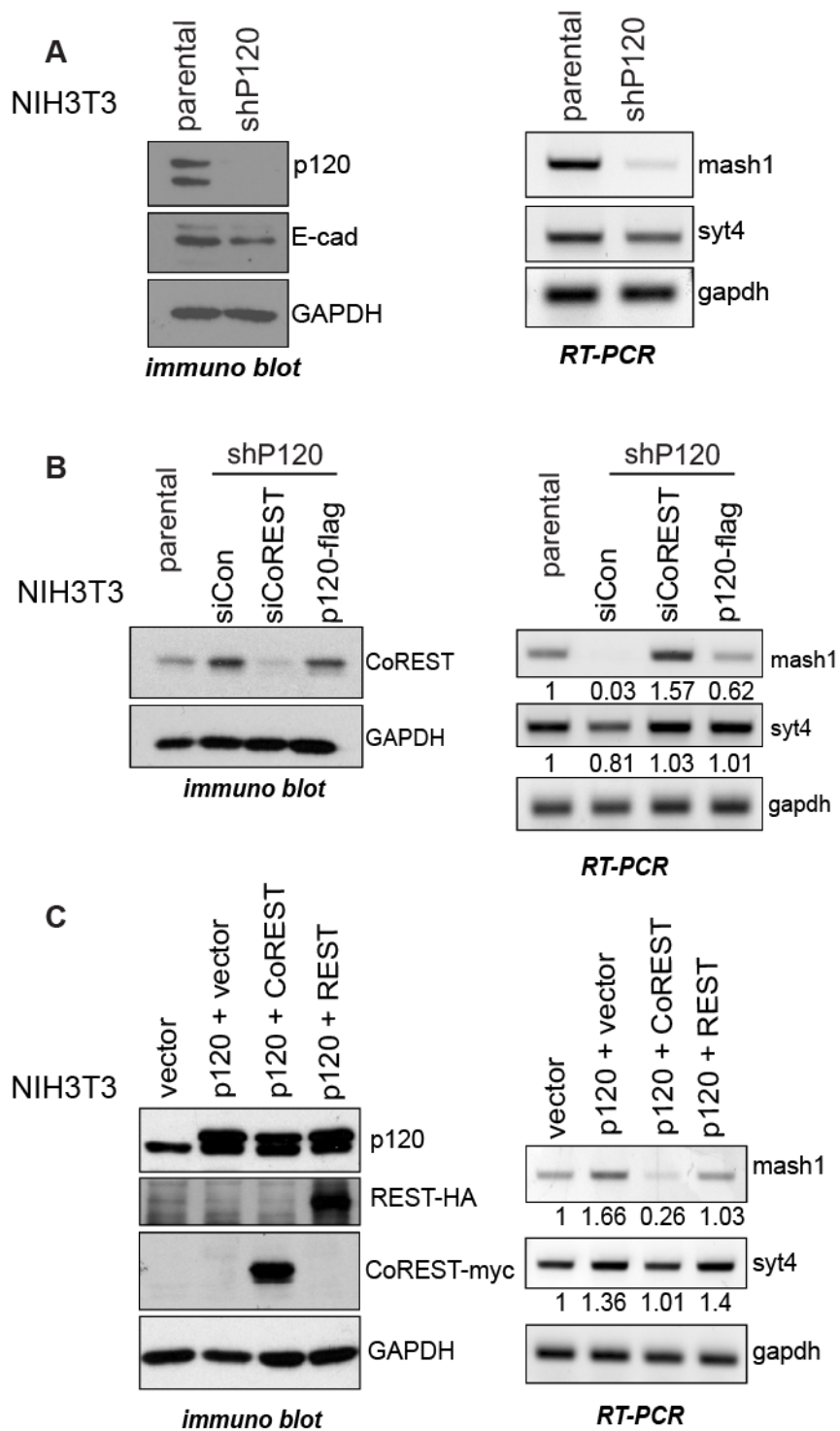


**Figure 28. P120-catenin destabilizes REST protein, likely through a proteasome-mediated mechanism.**

The indicated cDNA constructs were transfected into AB1-mESCs and 48hrs post-transfection, the cells were incubated with MG132 at 5mM final concentration for 2hrs before harvest. Exogenous REST and p120 proteins were detected using anti-HA and anti-Flag antibodies, respectively. See also Figure 27 for p120-catenin effects upon REST and CoREST protein levels.

*The effect of p120-catenin levels on REST/CoREST gene target transcription in other cell lines*

Using NIH3T3 and HEK293 cell lines, we tested whether p120-catenin modulated REST/CoREST gene targets. Consistent with p120 depletion effects in mESCs, we observed that p120 depletion in NIH3T3 cells decreased the transcript levels of mash1 and synaptotagmin4 (Figure 29A). As expected, the co-depletion of CoREST rescued p120 depletion effects on REST/CoREST gene targets in NIH3T3 cells (Figure 29B). Conversely, p120 expression increased expression from the indicated REST/CoREST target genes (Figure 29C), which was also observed in HEK293 cells (data not shown). These increases in transcript levels were completely rescued by CoREST expression or partially by the REST expression (Figure 29C). Our findings suggest that p120-mediated REST/CoREST gene target regulation extends beyond mESCs.



**Figure 29. P120-catenin modulates REST/CoREST gene targets in NIH3T3 cells.**

*A. P120-catenin knockdown decreases the expression of REST/CoREST gene targets in NIH3T3 cells. Using shRNA mediated knockdown, p120 was depleted in NIH3T3 cells. Anti-p120, anti-E-cadherin and anti-GAPDH antibodies were used for immunoblotting. The transcripts of REST/CoREST gene targets (mash1 and syt4) were tested by semi-qRT-PCR. Gapdh was used as an internal control.*

*B. P120 knockdown effects were rescued by CoREST knockdown.*

*CoREST was depleted using siRNA in p120-depleted NIH3T3 cells, using a liposome-mediated transfection method. Alternatively, p120 cDNA was transfected into p120-depleted NIH3T3 cells. 72hrs post-transfection, CoREST knockdown was confirmed by immunoblotting, and the transcript levels of the indicated REST/CoREST gene targets (mash1 and syt4) were measured by semi-qRT-PCR. The transcripts were normalized to gapdh and quantified relative to parental values.*

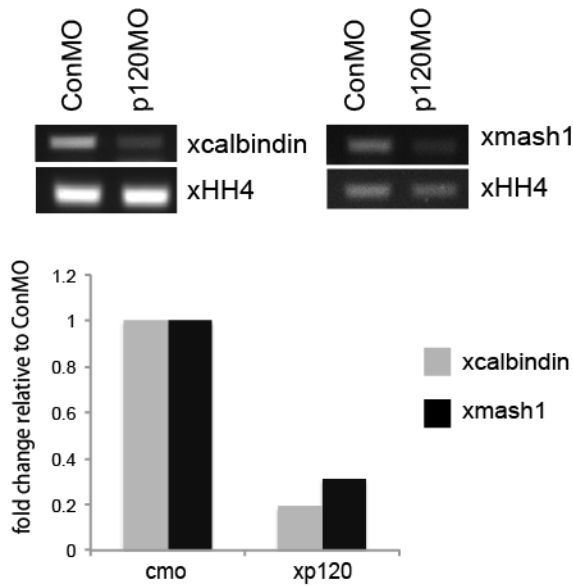
*C. CoREST expression rescues p120-catenin expression effects.*

*Using liposome-mediated transfection, p120-catenin cDNA plasmid was co-transfected with CoREST or REST cDNA constructs into NIH3T3 cells. 48hrs post-transfection, cells were harvested for immunoblotting or semi-qRT-PCR. Transcripts of the indicated REST/CoREST gene targets, mash1 and syt4 were normalized to gapdh and quantified relative to parental values.*



*P120-catenin depletion effects on REST/CoREST gene target expression in  
Xenopus laevis embryos*

To determine the functional effects of p120-catenin on the modulation of REST/CoREST gene targets in vivo, we conducted microinjection of an established morpholino antisense oligonucleotide to knock down p120-catenin (62) in *Xenopus laevis* embryos. We observed that p120 knockdown decreased transcripts of REST/CoREST gene targets, such as xcalbindin and xmash1 in embryos at gastrulation stage (stage 11) (Figure 30), suggesting that p120 modulates REST/CoREST gene targets across differential mammalian cell types and in amphibians.



**Figure 30. P120 knockdown increases repression of REST/CoREST gene targets in *Xenopus laevis* embryos.**

10 ng of morpholino antisense-oligonucleotide directed against xp120-catenin (p120MO) or standard-control morpholino (ConMO) was microinjected into both blastomeres of embryo at the 2-cell stage. The microinjected embryos were harvested at the gastrula stage (stage 11). *Xenopus calbindin* and *Xenopus mash1* transcripts were measured by semi-qRT-PCR, and *Xenopus histone H4* was used as an internal control.

## **Part Five: mESC stemness-marker expression is increased upon p120-catenin depletion.**

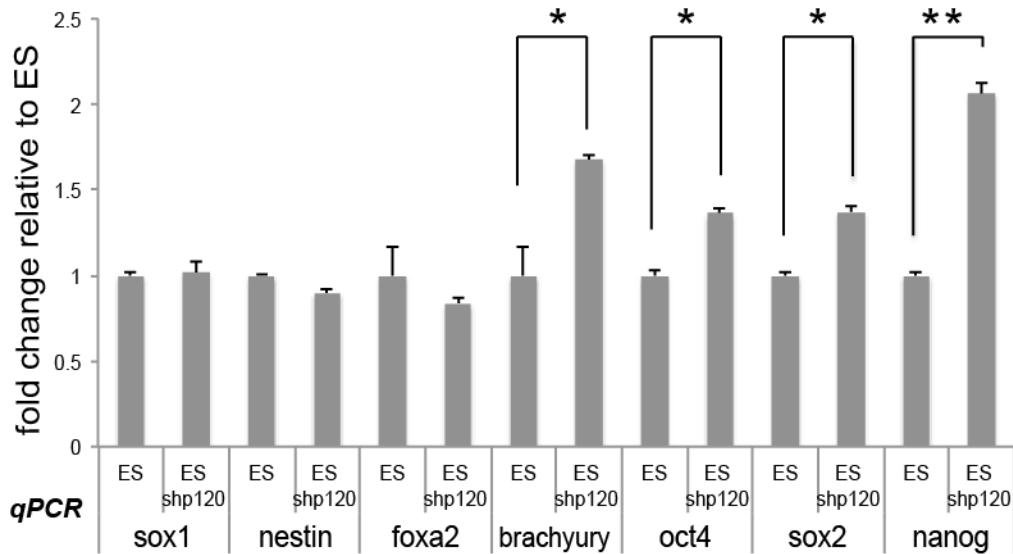
### *Novel roles of p120-catenin in mESC pluripotency and differentiation*

The REST/CoREST repressor complex contributes to preventing the precocious expression of gene targets involved in differentiation of neural stem/progenitor cells. Although controversial (24-26, 28), REST/CoREST may also play a role in maintaining pluripotency in mESCs. As reported, p120 additionally has nuclear roles apart from REST/ CoREST (43, 44). Using p120-depleted mESCs, we tested the expression of pluripotency and differentiation markers. We observed that the expression of nanog, one of the core pluripotency factors, was increased up to 2-fold upon p120 depletion (Figure 31). Although the transcription levels of other core pluripotency factors, such as oct4 and sox2, were modestly increased (Figure 31), oct4 and sox2 was significantly increased at the protein level in p120-depleted cells (Figure 32). We omitted immunoblotting data using anti-nanog antibodies because the anti-nanog antibodies did not work reliably in our hands even in pluripotent mESCs (data not shown). In addition to the molecular findings, we tested the p120 depletion effects on pluripotency using the alkaline phosphatase staining method. P120-catenin depletion did not alter mESC pluripotency based on alkaline phosphatase assays (data not shown). Further, p120 depletion did not affect the expression of differentiation markers such as sox1, nestin (neuro-ectodermal markers), foxa2 (endodermal marker), and brachyury (mesodermal marker) in mESCs (Figure 31).

Also, we observed that the transcriptional levels of brachyury and sox1 were slightly decreased upon p120 depletion (Figure 33). P120 expression conversely led to transcriptional decreases of Oct4, sox2 and nanog, by 20%-30% (Figure 33). The mechanism or significance of the differentiation marker modulation (eg. brachyury and sox1) in the p120 expression context remains to be further investigated. Although pluripotency marker expression was reduced upon p120 expression in mESCs, alkaline phosphatase readouts did not display a significant loss of pluripotency in mESCs maintained under pluripotency conditions (Figure 34).

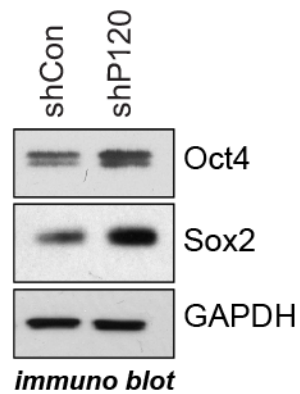
However, interesting effects arose when mESCs were placed under neuronal differentiation conditions. P120 expression in mESCs led to an obvious accelerated reduction in stemness relative to control mESCs (Figure 34). Consistent with the accelerated pluripotency loss upon p120 expression, p120 expression increased differentiation, as scored using doublecortin (DCX) expression (Figure 34). Also, in p120-expressing mESCs under differentiation conditions, decreased oct4 and sox2 protein levels supported our reduced alkaline phosphatase readouts (Figure 34). Taken together, our findings suggest that under differentiation conditions, p120-catenin affects the balance of pluripotency and differentiation, presumably in part by modulating expression of established pluripotency markers. That is, given we reveal that p120 binds REST and CoREST and that a functional relationship can be observed at

REST/CoREST gene targets, p120's effects upon pluripotency genes might in part arise as a consequence of p120's relationship with REST/CoREST.



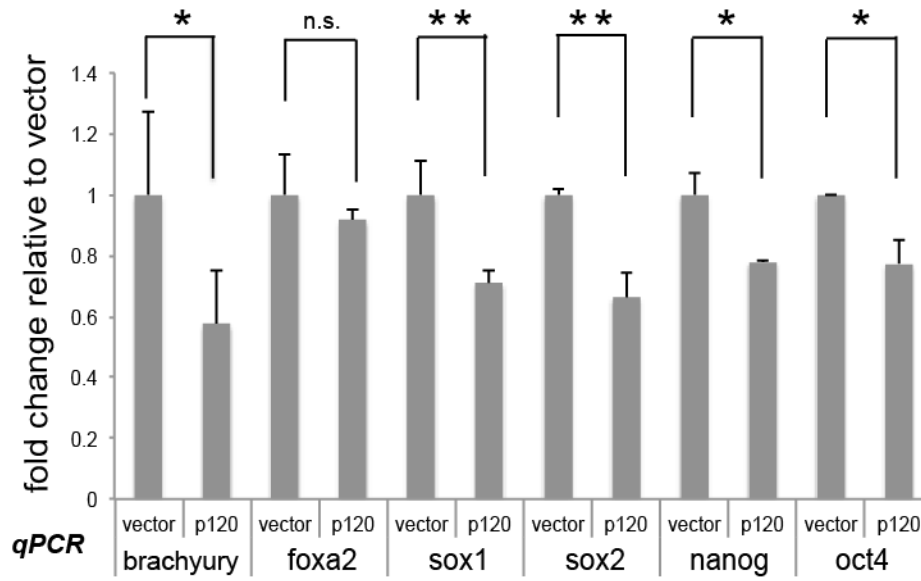
**Figure 31. P120-catenin depletion enhances transcription of pluripotency markers.**

Using control- and p120- depleted AB1 cells, the indicated pluripotency and differentiation marker transcription was tested by real-time PCR. (oct4, sox2, and nanog serve as pluripotency markers; sox1 and nestin as neuro-ectodermal differentiation markers; foxa2 as an endoderm marker; and brachyury as a mesoderm marker). Error bars represent SD. (One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ )



**Figure 32. P120-catenin depletion increases protein levels of the pluripotency markers Oct4 and Sox2.**

The p120-depleted and control mESC lysates, and anti-Oct4 and anti-Sox2 antibodies were used for immunoblotting. GAPDH was used as an internal control.

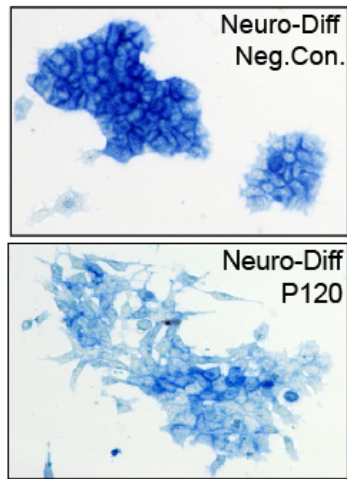


**Figure 33. P120-catenin expression decreases pluripotency marker expression.**

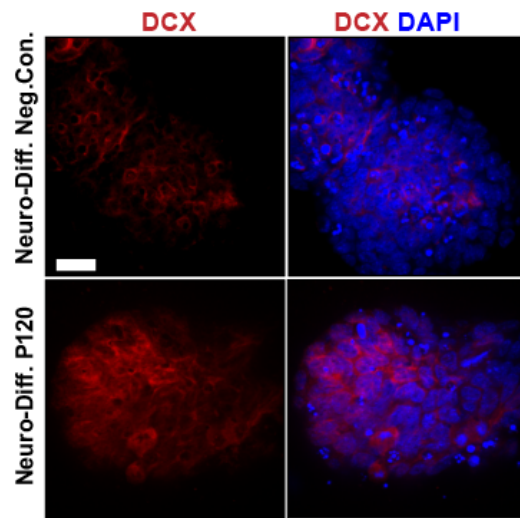
Vector and p120 cDNA constructs were transfected into AB1-mESCs. The transcript levels of pluripotency and differentiation markers were tested by real-time PCR. All transcripts were normalized to GAPDH. Error bars represent SD. (one asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ).



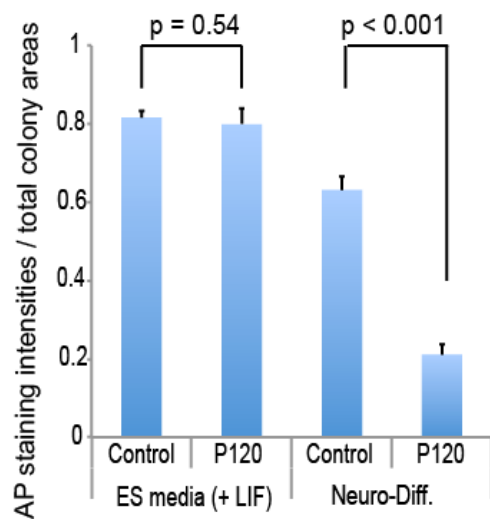
A



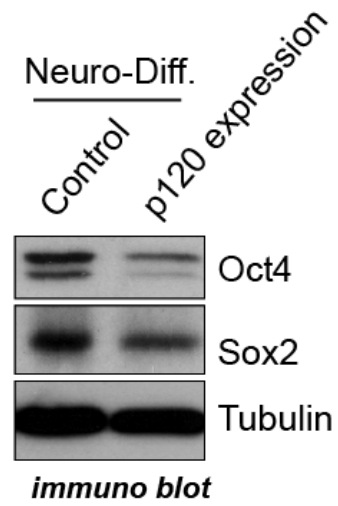
C



B



D



**Figure 34. P120 expression accelerates pluripotency loss and differentiation of mESCs under differentiation conditions.**

(A,B) P120-catenin stably expressing and control AB1-mESCs were maintained for 48hrs in stem cell media (containing LIF and at high serum concentration), or in N2B27 neuronal differentiation media. Using the alkaline phosphatase (AP) staining method, pluripotency was evaluated. Error bars represent SD. P values were obtained by Student t-test.

(C) Using control and p120-expressing AB1 cells, immuno-staining was performed with anti-DCX antibody. To induce differentiation, cells were maintained in N2B27 neuronal differentiation media for 72hrs. DAPI was used for counter-staining. Scale bar represents 30mm.

(D) Control and p120-expressing AB1 cells were harvested to detect Oct4 and Sox2 proteins. To induce differentiation, the cells were maintained in N2B27 neuronal differentiation media for 48hrs prior to harvest. GAPDH was used as an internal control.

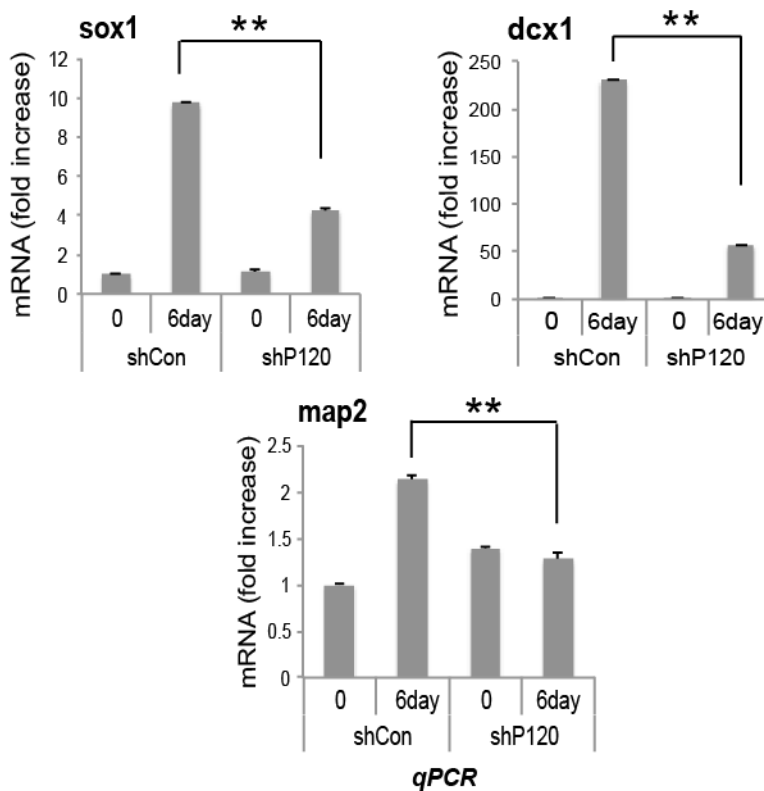
## **Part Six: P120-catenin modulates the neuronal differentiation of mESCs.**

Although some proteins of the p120-catenin subfamily were previously reported to play a role in neural development (63-67), p120-catenin's nuclear role has been unclear in the neural development context.

### *The p120-catenin depletion effect on neuronal differentiation of mESCs*

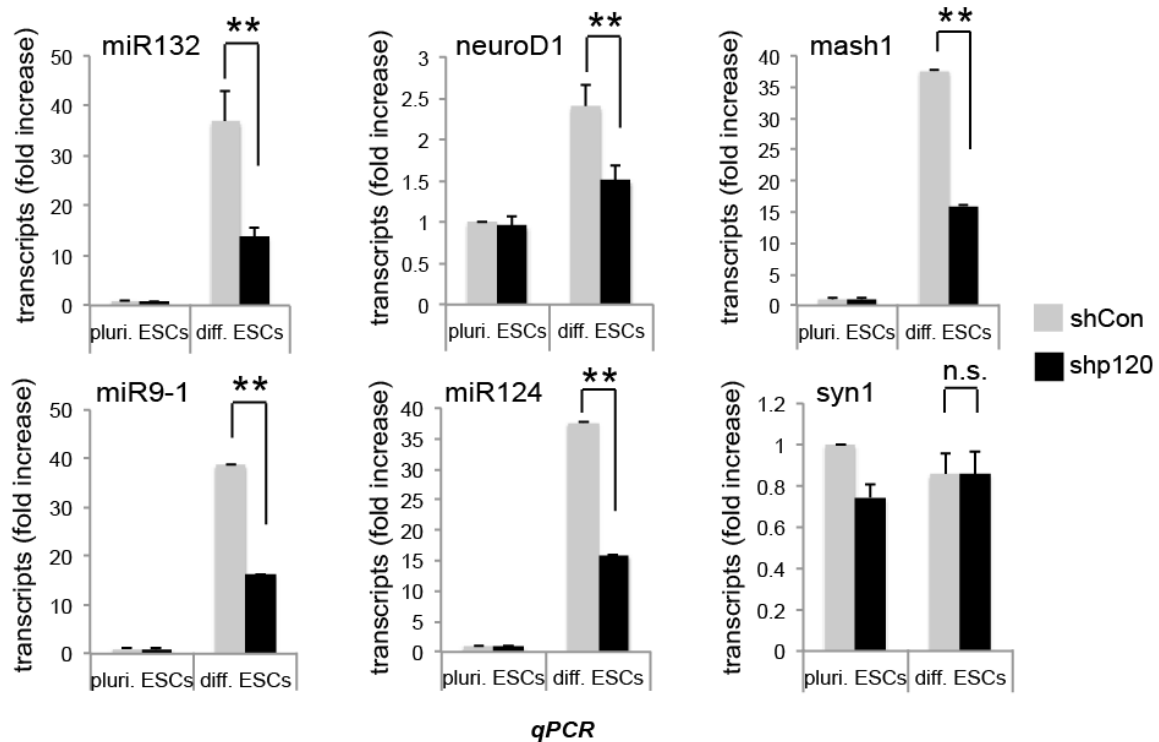
REST represses expression of multiple genes related to neuronal differentiation (8, 68). We assessed whether p120 modulates the neural differentiation of mESCs under neuronal differentiation conditions using the direct (monolayer) method. While the transcriptional levels of neuronal-differentiation markers such as *sox1*, *dcx1*, and *map2* were increased in control cells, p120 depletion decreased such neuronal differentiation marker expression under differentiation conditions (Figure 35). While the three neuronal differentiation markers we tested displayed a significant decrease in the transcription context, *dcx1* expression was graphically down-regulated (> 70%) upon p120 depletion. Since *dcx1* is a direct gene target of REST/CoREST, the p120 depletion effect on neuronal differentiation may be due to enhanced repression by REST/ CoREST in response to p120 depletion. Further, our findings that the transcriptional levels of *sox1* and *map2*, employed as general differentiation markers, also showed a significant response to p120 depletion, suggests that p120-catenin may play some role in the neurogenesis of mESCs.

To determine whether p120-catenin's effects on neuronal differentiation are related to enhanced repression of REST/CoREST gene targets, we tested the transcript levels of REST/CoREST gene targets that play a critical role in early neural development. P120 depletion decreased the transcript levels of REST/CoREST gene targets such as miR9-1, mash1, miR124, miR132, and neuroD1, consistent with the above results obtained from general neuronal differentiation marker expression (Figure 36). The p120 depletion effects were effectively rescued by co-depletion of CoREST (Figure 37). Also our REST ChIP-qPCR results had indicated that p120 depletion increased REST occupancy at the RE1 region of miR-124 relative to miR-124 5' UTR region (negative control). This reduced differentiation under differentiation conditions of mESCs subject to p120 knockdown may be a consequence of lessened displacement of REST from RE1 regions in gene targets (Figure 38).



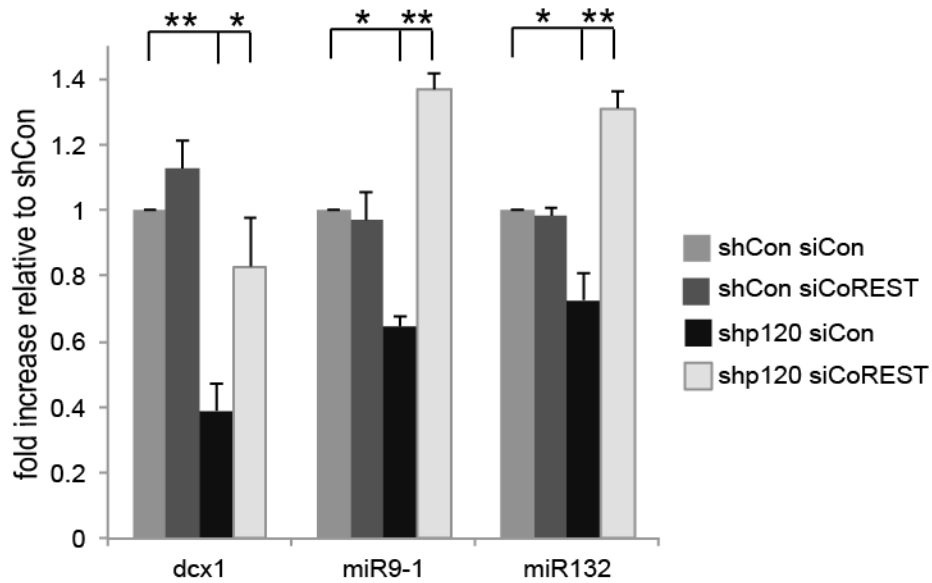
**Figure 35. P120-catenin depletion decreases neuronal differentiation marker expression in mESCs placed under neuronal differentiation conditions (direct/monolayer differentiation method).**

Control- and p120-depleted AB1-mESCs were maintained in N2B27 neuronal differentiation media for 6 days before harvest. The indicated neuronal differentiation markers were tested by real-time PCR. All transcripts were normalized to gapdh. Error bars represent SD. One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ . (Note: *sox1* serves as a neural stem/progenitor marker; *dcx1* as early neuronal differentiation marker; and *map2* as a late neuronal differentiation marker.)



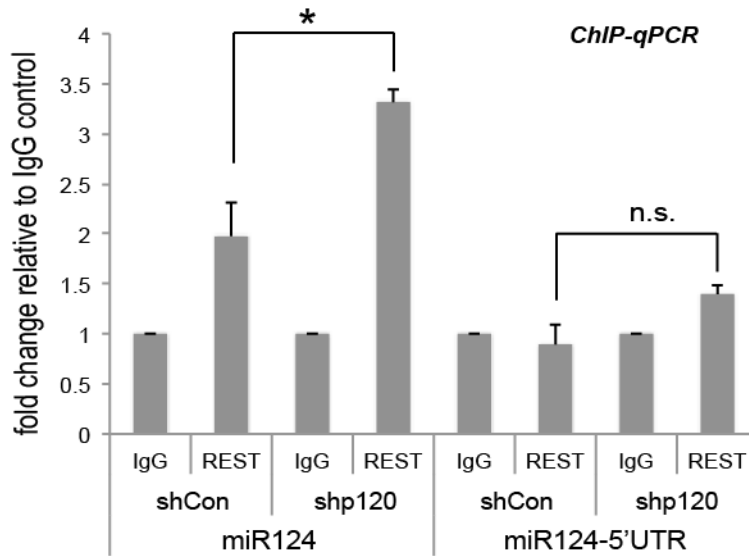
**Figure 36. P120-catenin knockdown decreases the transcription of REST/CoREST targets in mESCs subject to neuronal differentiation conditions (direct/monolayer differentiation method).**

P120- and control-depleted AB1-mESCs were maintained in N2B27 neuronal differentiation media for 4 days before harvest. The indicated REST/CoREST gene targets were tested by real-time PCR. All transcripts were normalized to gapdh. Error bars represent SD. (One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ; n.s., not statistically significant).



**Figure 37. CoREST co-depletion rescues p120-catenin depletion effects.**

CoREST was knocked down using siRNA in p120- and control-depleted AB1 cells. The cells were maintained in N2B27 neuronal differentiation media for 4days before harvest. The indicated transcript levels of REST/CoREST gene targets were tested by real-time PCR and normalized to gapdh. Error bars represent SD. (One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ).



**Figure 38. P120-catenin depletion increases REST binding to the miR-124 RE1 region in differentiating mESCs.**

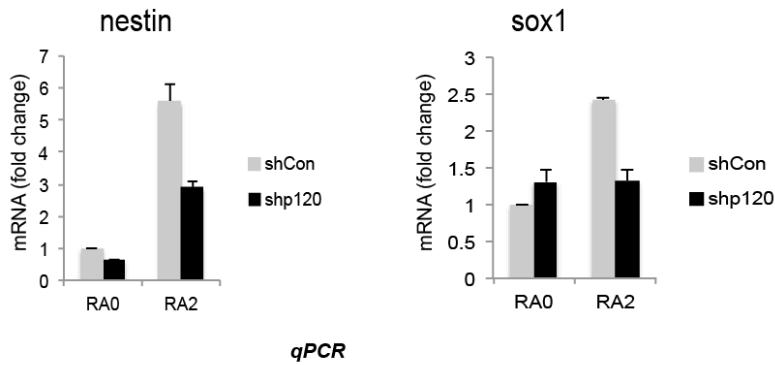
Control- and p120-depleted AB1-mESCs were maintained in N2B27 neuronal differentiation media over 4 days before harvest, and REST ChIP-qPCR was performed. The 5'UTR of miR124 was tested as a negative control. Error bars represent SD. (One asterisk,  $P < 0.05$ ; n.s., not statistically significant).



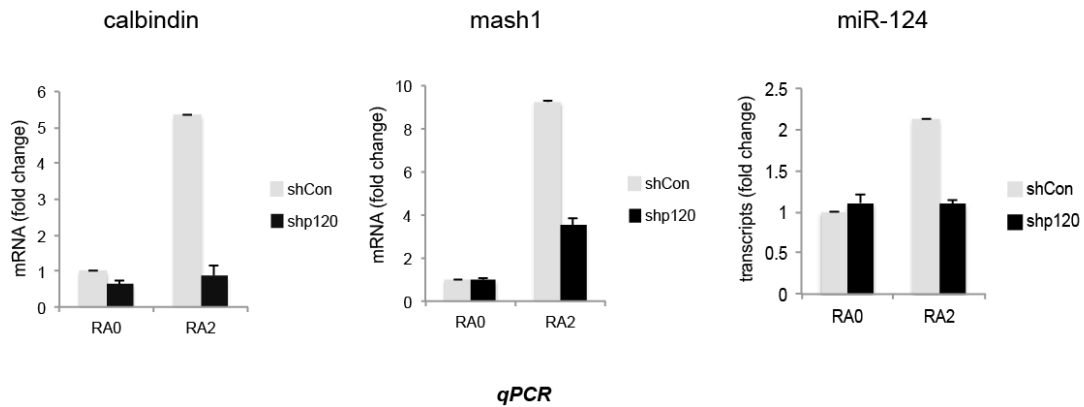
*Role of p120-catenin in mESCs differentiating under retinoic acid (RA) mediated neural differentiation*

To validate the role of p120-catenin in mESCs differentiating using the direct/monolayer neuronal differentiation method (see above), we applied another established neural differentiation method reliant upon retinoic acid (RA) (69). In RA-mediated differentiation, we observed that the mRNA level of nestin, a neural stem/progenitor marker was decreased upon p120 depletion (Figure 39A), in keeping with the down-regulated mRNA of sox1, another neural stem/progenitor marker, upon p120 knockdown in mESCs differentiating via the direct differentiation method (Figure 35). Likewise, the transcripts of REST/CoREST gene targets such as calbindin, mash1, and miR-124 were decreased in p120 depleted mESCs subject to RA-mediated differentiation (Figure 39B). Together, our findings obtained from two independent differentiation methods (direct/monolayer and RA-mediated) suggest that p120-catenin modulates differentiation of mESCs via modulation of the REST/CoREST complex.

A



B



**Figure 39. P120-catenin modulates the neural differentiation of mESCs.**

Using the established retinoic acid (RA)-mediated neural differentiation method, AB1-mESCs were differentiated. P120- and control-depleted AB1 cells were plated on bacterial culture dishes in DMEM including 10% FBS (but not LIF and beta-mercaptoethanol). Embryoid body formation over 4days was followed by an additional incubation for 2 days with RA before harvest. The transcripts of neural stem/progenitor markers (*sox1* and *nestin*) and REST/CoREST gene-targets (*calbindin*, *mash1*, and *miR-124*) were tested by real-time PCR. All transcripts were normalized to *gapdh*. (Note: RA0 = 4-day-old embryoid bodies; RA2 = 4-day-old embryoid bodies + 2-days RA treatment).

**Part Seven: Upstream pathway regulation: E-cadherin appears to modulate REST/CoREST gene targets via p120-catenin in mESCs.**

Thus far, our work has included resolution of a novel association of p120-catenin with REST/CoREST, and as a downstream effect, modulation of the balance of differentiation and pluripotency in mESCs subject to differentiation conditions.

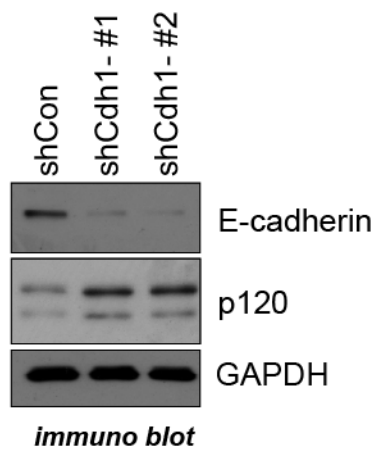
Now we concentrated on a key remaining question regarding the *upstream* modulator(s) of p120-catenin in the context of controlling REST/CoREST gene target expression.

Canonical Wnt-signaling has been reported to contribute to both stemness and differentiation in mESCs, and we earlier showed that canonical Wnt-signaling positively modulates p120-isoform1 (45). Based on this knowledge, we considered that Wnt signals may play an upstream role in positively regulating p120-catenin (isoform 1) and thereby REST/CoREST. However, in keeping with previous studies indicating that canonical-Wnt signals enhance stemness (70, 71), we found that upon incubation with Wnt1 and Wnt3a, the transcript levels of neural differentiation markers (sox1 and nestin), as well as REST/CoREST gene target (synaptotagmin4), were decreased as opposed to increased (data not shown).

We thus next turned our attention to cadherins. Compared to the reasonably well characterized roles of p120-catenin and E-cadherin in the context of cadherin

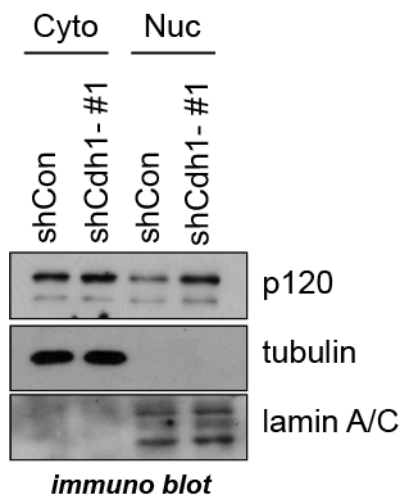
stability and adherens-junctions (39, 72, 73), their nuclear signaling roles in pluripotency and differentiation leaves much to be learned (31). However, some recent reports suggest a critical role of E-cadherin in the context of stemness. For example, reduced E-cadherin level is associated with the loss of pluripotency in human embryonic stem cells (hESCs) (74).

We tested the functional effects of E-cadherin levels on REST/CoREST gene targets in mESCs, as we predicted might occur via p120-catenin. Using shRNAs directly against E-cadherin, we generated E-cadherin depleted AB1 cells. Intriguingly, by an unknown mechanism, E-cadherin knockdown caused a modest increase in p120 protein levels (Figure 40). The E-cadherin knockdown effect was further interesting in increasing p120 protein levels in the nuclear fraction (Figure 41). As predicted if an enlarged signaling pool of p120 became available, E-cadherin depletion led to increased expression of REST/CoREST gene targets (Figure 42). We then tested whether E-cadherin knockdown effects on REST/CoREST target expression occur via p120-catenin. Indeed, we observed that p120 knockdown partially rescued the E-cadherin depletion effects, while the co-knockdown of beta-catenin (negative control) did not (Figure 43). These findings suggest that in an upstream capacity, E-cadherin plays a role in modulating the ability of p120-catenin to regulate REST/CoREST gene targets, presumably in turn modulating the balance of pluripotency and differentiation in mESCs.



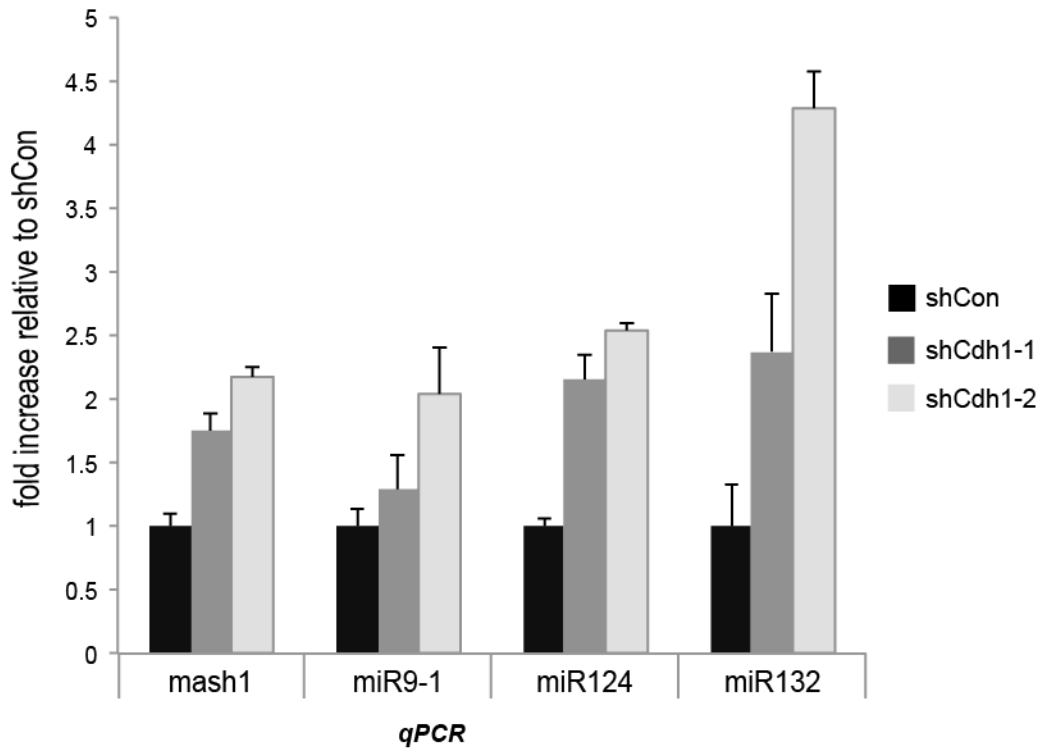
**Figure 40. P120-catenin protein levels increase in E-cadherin depleted mESCs.**

Using two independent shRNAs, E-cadherin was depleted in AB1 cells and whole cell lysates were then used for immunoblotting. Anti-E-cadherin and anti-p120 antibodies were used to detect the indicated proteins. GAPDH was used as an internal control.



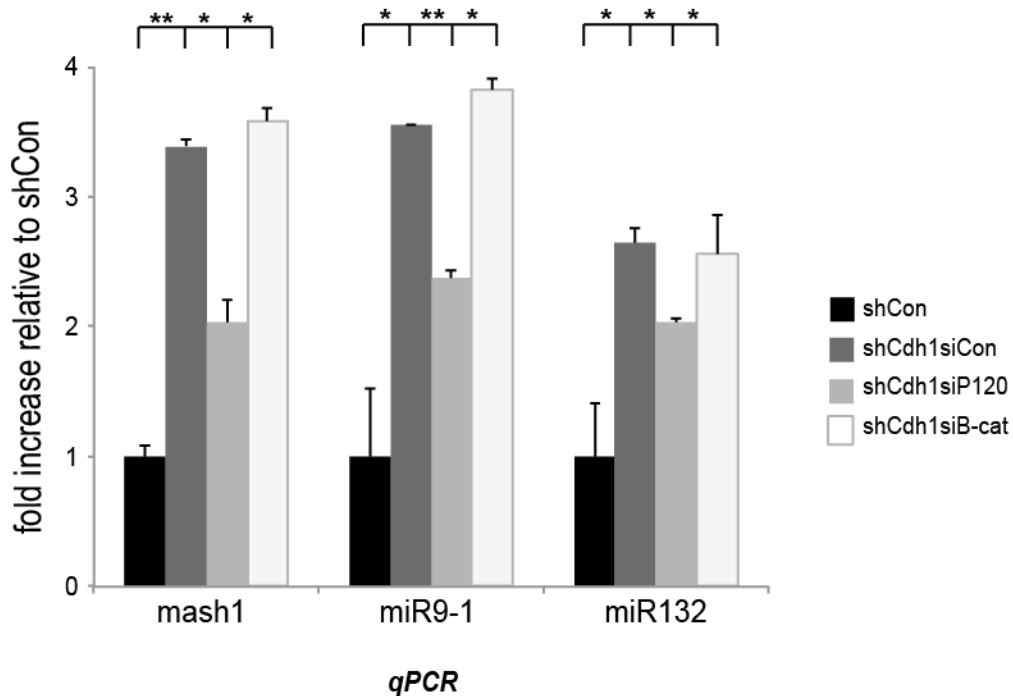
**Figure 41. P120-catenin's nuclear levels are increased upon E-cadherin depletion.**

Using a conventional subcellular fractionation method, cytoplasmic (Cyto) and nucleoplasmic (Nuc) fractions were obtained from E-cadherin- and control-depleted AB1-mESCs. The fractions were used for immunoblotting. (Note: tubulin serves as a cytoplasmic marker and nuclear lamin A/C as a nucleoplasmic marker).



**Figure 42. E-cadherin knockdown increases transcription of REST/CoREST gene targets.**

RNAs were extracted from control- and E-cadherin-depleted AB1-mESCs, and the indicated transcripts were tested by real-time PCR. All transcripts were normalized to gapdh. Error bars represent SD.



**Figure 43. P120-catenin knockdown (not beta-catenin knockdown) partially counteracts E-cadherin depletion effects.**

P120- or beta-catenin were knocked-down via siRNA-mediated RNAi in E-cadherin- or control-depleted AB1 cells. Transcripts of the indicated REST/CoREST gene targets were tested by real-time PCR. All transcripts were normalized to gapdh. Error bars represent SD. (One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ).



## **CHAPTER III**

## **DISCUSSION**

Pluripotency and lineage-specific differentiation are accurately modulated by multiple regulatory mechanisms. In embryonic and neural stem/progenitor cells, the REST/CoREST complex has been determined to play a role in preventing precocious gene expression, which is critical for precise initiation of differentiation as well as lineage-specific differentiation. For example, in pluripotent embryonic stem cells, multipotent neural stem/progenitor cells, and differentiated non-neural cells, REST/CoREST contributes to suppressing gene targets required for neural differentiation (8, 20-22, 75, 76). Whereas a fair amount of work has indicated how REST/CoREST regulates gene targets in embryonic and neural stem cells and in differentiation, upstream mechanisms regulating REST/CoREST have been less clear. A previously reported mechanism is the betaTrCP E3 ligase ubiquitinates REST, leading to its proteasomal degradation in neural stem cells (19). Likewise, REST is down-regulated in neural-differentiating ESCs through proteasome dependent REST destruction (18).

In this study, we showed that p120-catenin regulates the repressive function of the REST/CoREST complex in both pluripotent and differentiating mESCs via p120 association with REST and CoREST. Although further studies are needed to fully understand p120's downstream actions in nuclear REST/CoREST modulation, we also observed that E-cadherin regulates the novel p120:REST/CoREST pathway more upstream.

*P120-catenin modulates occupancy of REST/CoREST at DNA.*

In modulating REST/CoREST gene targets, we observed that p120 associates with both REST and CoREST in different contexts, for example, using in vitro binding as well as endogenous co-immunoprecipitation tests. Further, mapping studies showed that p120's central Armadillo domain associates with both REST and CoREST, and the zinc-finger (DNA binding) region of REST associates with p120. While either REST or CoREST can form a complex with p120 in an independent manner, we observed that p120 can also simultaneously form a complex with REST and CoREST, suggesting that p120 may affect REST/CoREST gene targets through modulating REST, CoREST or both. For example, p120 affects REST's ability to modulate REST/CoREST gene targets by directly competing with DNA for REST binding. The direct competition model is supported by EMSA. P120-catenin not only displaces REST from preformed REST:DNA (RE1) complexes, but also prevents REST:DNA (RE1) complex formation upon prior p120:REST association. Likewise, the direct competition model is consistent with a specific interaction between the zinc-finger region of REST and p120-catenin, supporting the idea that REST's association with DNA may be prevented by prior p120-catenin docking to the zinc-finger region of REST. Moreover, a displacement mechanism appears similar in concept to p120's removal of Kaiso from its DNA (KCS) consensus sites, with Kaiso being a zinc-finger repressor (44) in which p120's association on either

side of Kaiso's zinc finger region leads to its dissociation from sequence-specific KCS sites in gene control regions (60).

In addition to the direct effect of p120-catenin competing with DNA for REST binding, p120 may indirectly affect REST binding to DNA through an impact upon CoREST's association. Some components of the larger repressor complex, such as CoREST and Brg1, appear to stabilize REST's association with DNA by associating with other DNA-binding proteins (16, 77). Indeed, the association of REST and CoREST in vitro was decreased in the presence of p120's Armadillo domain (data not shown). REST ChIP experiments also supported p120's modulation of REST occupancy at DNA. Expression of p120's central Armadillo domain, but not its amino-terminal domain, led to a significant decrease of REST occupancy at RE1 consensus sites of gene targets.

Another possibility to consider in p120-catenin's mechanism of action, and why it may bind both REST and CoREST, is that it first associates with CoREST within the REST:CoREST:DNA complex. Especially if this initial interaction is of relatively low affinity, it would put p120 in better proximity to then displace REST from DNA, because it would greatly increase its local concentration. Since REST recruits a variety of nuclear proteins to RE1 sites and forms a bulky repressor complex with DNA, REST's zinc-finger region may also be relatively sterically inaccessible to p120-catenin in vivo. Like most DNA binding factors, REST is likely to have an on-and-off or "breathing" form of residence on its (RE1) consensus binding site. Therefore, p120-catenin might use CoREST as a form of initial staging area to enhance access to REST's zinc-finger domain that has

briefly dissociated from DNA, and prior to its recapturing its consensus binding site. This local hand-off mechanism for p120 may enhance the probability for it to sterically prevent REST's reassociation with DNA, leading to gene derepression (activation).

Although more detailed studies will be necessary to clarify the mechanisms of p120:REST/CoREST modulation, we surmise that p120 diminishes the ability of the REST/CoREST complex to associate with DNA and/or other gene-regulatory protein factors in a direct (competition for REST) - and/or an indirect (associating with CoREST) - fashion.

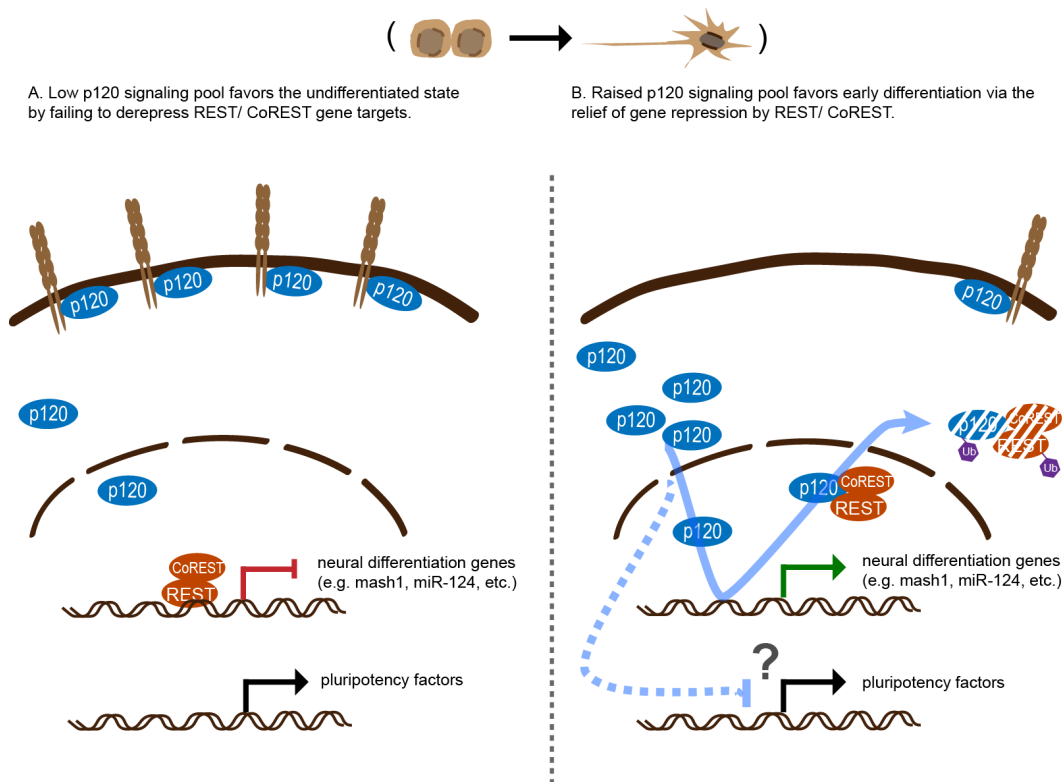
In addition to the REST/CoREST displacement model, the increased turnover of REST and CoREST in the presence of p120-catenin may contribute to gene activation. More studies should be likewise be performed to address p120-mediated post-translational regulation of REST and CoREST, as we observed that p120 decreases REST/CoREST protein stability and enhances their proteasome-mediated destruction. Thus, following the displacement of REST/CoREST by p120, REST/CoREST might be translocated to the cytoplasm (along with p120) to enhance destruction of the complex (18). Possibly, both the displacement and turnover of REST/CoREST as mediated by p120-catenin may contribute to a more effective activation of REST/CoREST gene targets.

#### *P120-catenin modulation of gene targets*

We showed that although not all REST/CoREST gene targets were affected by p120 depletion, multiple REST/CoREST gene targets, in particular, genes such as mash1, miR124, miR9-1, and miR132 that are highly expressing in neuronal tissues such as brain, were strongly repressed by p120 depletion in mESCs. Our results appear to be consistent with previous reports that a cohort of neuronal genes, including some of those harboring canonical RE1 sites, selectively do not respond to REST depletion in mESCs (75). Since miR-124 and miR-9 directly repress expression of CoREST and REST as part of a negative feedback loop (78, 79), the observed increase in REST and CoREST protein levels following p120 depletion may in part result from the lowered expression of miR-124 and miR9-1.

This effect would be in addition to increased protein stability of REST and/or CoREST in response to the knockdown of p120, the depletion of which leads to their reduced delivery of REST/CoREST to the proteasome in our model. We observed that decreased protein levels of REST and CoREST were restored by proteasome inhibitors, suggesting that p120 modulates protein degradation of REST and CoREST in keeping with a previous report on REST ubiquitination and destruction (19). Likewise, p120-isoform1 protein is down-regulated by ubiquitination and protein degradation (45), and p120-isoform1 shares the same E3 ligase (beta-TrCP) with REST (19), suggesting that other components associating with p120:REST might share an analogous post-translational modification and destruction mechanism.

It remains to be more fully studied the mechanisms by which REST and CoREST proteins are destabilized by p120-catenin. However, in terms of REST/CoREST target gene modulation, our findings of p120's biochemical association with REST/CoREST, as well as of p120's effects upon REST:DNA interactions, are consistent with the de-repression (activation) of REST/CoREST gene targets by p120 displacing REST from DNA gene-control regions. We describe our model in Figure 44.



**Figure 44. Working model of p120's modulation of mESC differentiation**

A. In pluripotent mESCs, although a small fraction ("signaling pool") of p120-catenin is present inside cells away from cell-cell borders, a considerably larger fraction of p120-catenin is sequestered to junctional regions. In this scenario, the REST/ CoREST complex suppresses precocious expression of their gene targets.

B. In early differentiating mESCs, decreased E-cadherin level enlarges p120's signaling-pool in the cytoplasm, leading to the de-repression of REST/CoREST gene targets. The transcriptional activation of such gene targets contributes to lineage-specific differentiation of mESCs. Furthermore, pluripotency-factors such as Nanog, Oct4 and Sox2 are decreased upon the increase in p120-catenin's signaling pool, which might favor ESC differentiation by an unknown mechanism(s).



*P120-catenin in modulating stemness and differentiation.*

Although there is disagreement in the field regarding the role of REST in maintaining stemness of embryonic stem cells (24-28), REST has been suggested to maintain the balance between pluripotency and lineage specific differentiation in both embryonic and neural stem cells (20-22, 80). This is based upon REST's direct modulation of differentiation-promoting gene targets, in particular those relevant to neural differentiation. Knowledge on the mechanisms by which REST modulates its targets is therefore needed to understand both normal development and pathological processes. For example, REST/CoREST's uncontrolled repressive activity contributes to undesired gene silencing in some disease states. REST's abnormal expression is implicated in the altered levels of non-coding RNAs (81). In particular, through repression of miR-124 expression (and increased expression of miR-124 targets), the ectopic expression of REST increases the self-renewal and tumorigenic potential in glioblastoma multiforme (82). In human glioblastoma patients, high expression of REST is associated with greater tumorigenic potential and worse prognosis, such as being correlated with shorter survival rates and refractory periods following chemotherapy (83, 84)

In order to maintain stemness as well as respond rapidly and precisely to differentiation cues, ESCs are thought to be kept in a “poised” condition in which core pluripotency factors such as Oct4, Nanog, and Sox2 occupy genes encoding differentiation proteins and ncRNAs (1). Since the REST/CoREST

complex suppresses differentiation genes in ESCs, our findings suggest the possibility that p120 may assist in releasing the REST/CoREST complex in some gene contexts to promote differentiation in response to differentiation signals. Because we observed that a portion of p120-catenin locates to the nucleus of pluripotent mESCs prior to differentiation, p120 may speculatively be pre-associated with the REST complex, or another type of complex, to assist in maintaining readiness (poised state) for differentiation cues. We will discuss E-cadherin as a possible upstream determinant affecting p120's nuclear function (see below).

While RNAi mediated p120 knockdown lowered the expression of REST/CoREST target genes encoding neural differentiation proteins and miRNAs, p120 knockdown did not completely suppress neuronal differentiation in neuronal differentiation conditions. Thus, although shRNAs directly targeting p120-catenin significantly decreased expression of p120, thus resulting in increased repression of multiple REST/CoREST gene targets, the expression of REST/CoREST targets that is retained appears to be adequate for differentiation to proceed. Although speculative, given that other p120-subfamily members exist, such as ARVCF- and delta-catenin that associate with REST and/or CoREST, there may be functional redundancies. Thus, the co-depletion of p120 and delta-catenin (etc.) may contribute to more effective suppression of REST/CoREST target gene expression, and thus more effectively block differentiation of mESCs when subject to neuronal differentiation conditions.

Our findings using mESCs appear to be inconsistent with a previous study using hESCs, where p120-catenin depletion resulted in loss of pluripotency under hESC maintenance conditions (74). However, it should be noted that mESCs differ from hESCs in a few critical aspects (85). As an example, cell-cell dissociation does not lead to pluripotency loss in mESCs (86). P120-catenin is necessary to stabilize E-cadherin, and decreased cadherin levels influence cell-cell interactions (87). As opposed to hESCs, we did not observe that p120 knockdown led to differentiation of mESCs. Moreover, the depletion of E-cadherin in mESCs did not display enhanced differentiation, whereas we found that E-cadherin depletion generated smaller sized colonies. This finding is in keeping with previous mESC studies (88). Taken together, although a role of REST/CoREST in hESCs was not evaluated in the prior report, our mESC results might be compatible with their hESC findings, considering the differential characteristics of mouse versus human ESCs.

#### *E-cadherin, a potential upstream modulator of p120-catenin nuclear activity*

Since the canonical Wnt signaling pathway had been indicated to regulate REST expression (89), we asked if canonical-Wnt ligands modulate REST/CoREST gene targets through p120-catenin in mESCs. However, given that the REST/CoREST gene targets we tested showed little response to canonical-Wnt ligands (data not shown), we instead went on to investigate the impact of cadherin-catenin complex in regulation of p120 and downstream REST/CoREST.

Although cell-cell interactions play a more apparent role in fate decisions (eg. pluripotency versus differentiation) in hESCs rather than mESCs (74), we looked for potential functions of E-cadherin in p120-mediated modulation of REST/CoREST gene targets in pluripotent mESCs. Prior studies have shown that E-cadherin levels dwindle in the neuroectoderm of early mouse embryos (29). This allowed the possibility that the signaling pool of p120-catenin may become enlarged due to p120's lessened sequestration by E-cadherin (90, 91). That is, an expanded p120-catenin pool might more effectively de-repress (activate) REST/CoREST gene targets. Indeed, as predicted in this model, E-cadherin depletion led to the increased expression of REST/CoREST gene targets. As determined upon p120 co-depletion (rescue analysis), p120-catenin at least in part contributed to the E-cadherin depletion effects upon REST/CoREST modulation. Together, our findings indicate that the E-cadherin-p120-catenin complex participates in gene-control, and that the loss of E-cadherin appears to relieve suppressed REST/CoREST gene targets via the action of p120-catenin. Considering that delta-catenin is also capable of binding CoREST as mentioned earlier, delta-catenin might play an additive role with p120-catenin in REST/CoREST modulation.

E-cadherin protein levels were steadily lowered during neuronal differentiation of mESCs. Conversely, N-cadherin protein became apparent at 4 days after initiating neuronal differentiation, and continued to increase during differentiation (data not shown). These observed expression patterns are compatible with

former findings in which N-cadherin expression was found to be critical for development and the functions of neuronal cells (92, 93). Since we observed that differentiating p120-depleted mESCs displayed lowered N-cadherin protein levels (unpublished data), one might question if p120 depletion led to impaired neuronal differentiation not directly via REST/CoREST modulation but instead indirectly via reduced cell-cell interactions. Arguing against the latter possibility, is that the expression of a p120 mutant (N478A) that is incapable of binding cadherin remained able to rescue REST gene targets in p120 depleted mESCs. Thus, it seems that it is p120-catenin as a signaling mediator rather than as a stabilizing component in cadherin-mediated cell-cell adhesion that is mainly responsible for the observed effects. None-the-less, given the complexity of neural development (etc.), we expect that p120 is likely to modulate neuronal differentiation in both cadherin-dependent and independent manners, as well as REST-dependent and independent manners.

### *Concluding remarks and Future directions*

Junctional complexes including those of the adherens junction are essential to maintain tissue homeostasis and functions. As a key component, p120-catenin contributes to stabilizing adherens junction at cell-cell border and to modulating the cell cytoskeleton at this as well as other cytoplasmic regions. In addition to p120's junctional and cytoskeletal roles, novel nuclear roles have been steadily uncovered. Here, our finding of the REST/CoREST complex as a new p120-catenin's binding partner will assist in extending our knowledge of the role of p120-catenin in the nucleus.

Given the accepted relevance of REST/CoREST in pluripotency and differentiation, our findings of p120 in relation to REST/CoREST should in time provide insights on development and potentially diseases such as cancer. Previously, we showed that Dyrk1a (Dual specificity tyrosine-phosphorylation-regulated kinase 1A) stabilizes p120-catenin in mammalian cells and vertebrate model systems such as *Xenopus laevis* (94). In the Down syndrome mouse model, Dyrk1a decreases REST levels, resulting in dysregulated pluripotency and precocious differentiation of the derived mESCs (95). As the mechanism of Dyrk1a's modulation of REST at the protein level remains unknown, we are interested in the possibility that p120-catenin may serve as the intermediary. One potential model is that increased Dyrk1a levels during neural differentiation (96), may favor p120-catenin protein stability. Based upon our findings, this stabilized

p120-catenin would be expected to enhance REST/CoREST degradation following their displacement from DNA. Such a possibility requires testing, but might suggest an indirect means by which Dyrk1a modulates REST/ CoREST in Down syndrome.

REST expression is strictly and differentially regulated in accordance with the context. Tight regulation of REST expression is essential, with deregulated REST being implicated in the initiation of progression of multiple cancers. For example, the loss of REST expression in non-neural tissues such as breast, lung, and colon is thought to favor tumorigenesis (97, 98). Since p120-catenin lacking exon B (encoding an NES/ Nuclear Export Signal) has been observed in some tumorigenic tissues (e.g. colon, pancreas, prostate), excessive nuclear p120-catenin may perturb REST functions by de-repressing (activating) oncogenic REST targets such as TrkC (99).

In contrast to its role as a tumor suppressor in non-neural tissues, REST plays an oncogenic role in brain tumors. The aberrant expression of REST is implicated in medulloblastoma (100, 101), as well as glioblastoma multiforme (GBM) (82, 83). However, REST dysregulation alone may be insufficient, whereas when occurring in combination with aberrant c-myc expression, cerebellar tumors are induced (102). Given that c-myc's transcriptional expression is positively modulated by p120's de-repression of the kaiso repressor (47), it is conceivable that p120-catenin promotes brain tumors through effects upon the target genes of both REST and kaiso. Therefore, more in-depth examination of the

relationship between p120-catenin and the REST/ CoREST complex in a tumorigenic context may ultimately offer opportunities to better understand certain forms of cancer.

It will also be important to examine roles of the p120:REST/CoREST complex in an in vivo system, including in stemness/ differentiation contexts. Not only is REST critical in neurogenic and eye development (103-105), p120-catenin likewise contribute to eye and craniofacial development (as do other catenin subfamily members such as ARVCF-catenin) (67, 106, 107). Since we recently observed that XcoREST also plays an essential role in eye and head development through loss-of-function experiments (unpublished data), it will likely prove informative to look for functional interactions between p120-catenin, REST and CoREST in developmental contexts. For example, using animal models such as *Xenopus laevis*, we could undertake loss- and gain-of-function approaches in conjunction with rescuing experiments. It is conceivable that the phenotypes generated by knockdown or over-expression of REST and CoREST would be rescued by p120 knockdown or co-expression, respectively.

Lastly, given the association of REST/CoREST with chromatin regulatory factors such as lysine-specific histone demethylase 1A and histone deacetylases (e.g., HDAC1 and HDAC2), later studies will likewise be needed to flush out molecular details to better account for the observed outcomes. Further, while not examined here, CoREST modulates many genes in which REST is not involved; for



example, it additionally associates with gene control regions through transcription factors such as ZNF198 (108). Thus, p120's effect on gene regulation may be wider still, and perhaps coordinated with its previously reported regulation of other transcription factors, such as Kaiso and Glis2 (43, 44). Thus, genome-wide studies using next generation sequencing methodology may broaden understanding of p120-catenin mediated gene modulation, by uncovering novel nuclear proteins associating with p120-catenin and putative gene targets.

Overall, my study revealed a new role of p120-catenin in mESC differentiation, and identified novel binding partners of p120-catenin. This work has thus been towards addressing the roles of p120-catenin subfamily members in the nucleus, and further supports the view that multiple catenins as opposed to beta-catenin alone make vital contributions in vertebrates.

## **CHAPTER IV**

### **MATERIALS AND METHODS**

### cDNA Cloning and Plasmids

Mouse p120-catenin (p120-1B), mouse CoREST, and mouse REST cDNA constructs were purchased from Openbiosystems and sub-cloned into the pCS2 vector. For p120-catenin stable expressing cell generation, the mouse p120-catenin cDNA was sub-cloned into pFlag-CMV vector (Sigma). To generate NLS-mutant p120, KK residues (K622 and K623) of mouse p120-catenin were mutated to AA using site-directed mutagenesis (Invitrogen). For interaction mapping studies, p120-catenin fragments were cloned as follows: the amino-terminal fragment corresponds to all p120 amino acids prior to Armadillo-repeat 1; the Armadillo-repeat fragment contains repeats 1 through 8.5; and the carboxyl-terminal tail fragment was generated from Armadillo repeat 8.5 through p120's carboxyl-terminal amino acid. P120 cDNA fragments were generated using PCR-based cloning into pCS2. All fragments of REST and CoREST for interaction-domain mapping tests were generated by PCR-based cloning into pCS2.

### Cell Culture and Transfection

HEK293 and NIH3T3 cells were cultured in complete medium (DMEM with 10% fetal bovine serum [FBS] and antibiotics) following standard protocols. AB1 mESCs (kindly provided by Dr. Michelle Barton) and TC1 mESCs (kindly provided by Dr. Jan Parker-Thornburg) were grown on feeder cell-free and gelatinized dishes either in DMEM containing 1000 U/ml LIF, 20% FBS (ES-qualified, Invitrogen), antibiotics and beta-mercaptoethanol or in 2i stem cell

media containing DMEM/F12, neurobasal media, N2, B27 supplements, LIF, CHIR99021 (final 3uM), PD0325901 (final 10uM), antibiotics and beta-mercaptoethanol. Media were replaced every day and sub-culture was done every two days to prevent stem cell colony overgrowing. Accutase was used to detach cells. For plasmid transfections, cDNA-constructs were transfected into cell using lipofectamine2000 (Invitrogen). For siRNA oligonucleotide transfections, lipofectamine RNAiMAX reagent (Invitrogen) was used. SMARTpool-siGENOME siRNAs against mouse p120, ARVCF, CoREST, E-cadherin and REST were purchased from Dharmacon.

#### Viral Transduction and Selection

For viral packaging, 293T cells were plated on 100mm dishes. When grown at 50% confluent, cells were transfected with psPAX2, pMD2.G, and selected lentiviral shRNA constructs. The media including virus were obtained 48hrs and 72hrs after transfection, and saved at -80C deep freezer. During infection, polybrene was added to the plated cells, and 48 hrs after infection, cells were subjected to puromycin selection at 1.5ug/ml of puromycin concentration for 48-72 hrs, and selected cells were maintained in puromycin including media. The indicated shRNAs were purchased from the ShRNA and ORFeome Core at The University of Texas MD Anderson Cancer Center (Houston, Texas).

### In Vitro Neuronal Differentiation

To generate neuronal differentiation in vitro from mESCs attached on dish, we followed previously published protocols (109). AB1 and TC1 cells were plated on gelatinized dishes in N2B27 neuronal differentiation medium (1:1=DMEM/F12 with N2 supplement and L-glutamine:neurobasal medium with B27 supplement; Invitrogen). Media were replaced every day. Differentiating cells were harvested at the indicated times for further experiments. To generate neuronal differentiation we also used a well-defined retinoic acid (RA) -mediated neuronal differentiation. The trypsin treated ES cells were plated on low attachment petri dishes with DMEM including 10% FBS and antibiotics (but not including LIF and beta-mercaptoethanol) to form embryoid bodies (EBs) and maintained for 4days. Media were replaced every other day. 4 days after EB formation, media were replaced with RA containing media and EBs were grown for another 4days.

### In Vitro Transcription and Translation and In Vitro Binding Assay

In vitro binding assays were performed as previously described (110). In brief, proteins were synthesized using the TNT SP6 high-yield wheat germ protein expression system (Promega). To conduct in vitro binding assays, we incubated synthesized proteins with the indicated antibodies in PBS containing protease inhibitors and 0.5% Nonidet P-40 for 1hr and precipitated them using Protein A/G plus Agarose (Santa Cruz) by additional incubation for 1hr. Immuno-complexes were washed three times with 0.5% Nonidet P-40 containing PBS.

### Glutathione S Transferase (GST) Pull-down Assays

GST pull-down assays were conducted using a previously described protocol (52). In brief, the indicated cDNA plasmids were transformed into BL21(DE3) competent cells. The transformed cells were grown at 37C overnight. 10ml of cultured LB broth was added to 200ml of LB media and cultured at 37C until it reached an OD600 of 0.6. Then 1M stock of IPTG was added to a final concentration of 0.2mM, and cells were induced at 30C for 3hrs. Bacterial cell pellets were resuspended in PBS including PMSF and broken by sonication.

Glutathione Sepharose 4B beads (GE Healthcare) and Amylose Magnetic beads (New England Biolabs) were used to precipitate GST- and MBP- fusion proteins, respectively. Bacterially purified GST-CoREST or REST proteins (GST cleaved from REST using thrombin protease) were incubated with bacterially purified MBP fusion to p120 protein or protein fragments and precipitated using glutathione Sepharose-4B resin. Co-precipitation was further analyzed using SDS-PAGE and immunoblotting.

### Nuclear Fractionation, Endogenous Co-Immunoprecipitation, and Immunoblotting

Conventional protocols were used for nuclear fractionation (111). We followed standard immunoprecipitation protocols, and diluted nuclear fractions or whole cell lysates were used for co-immunoprecipitation. We used 2 ug of anti-CoREST or anti-REST (Millipore) antibodies for immunoprecipitation, along with SDS-PAGE and immunoblotting. The antibodies used for immunoblotting were as follows: anti-p120, anti-E-cadherin (BD Transduction Laboratories), anti-GAPDH

and anti-tubulin (Santa Cruz), anti-myc (9E10), anti-HA epitope (Developmental Studies Hybridoma Bank), anti-actin, and anti-Flag (Sigma), anti-lamin A/C (Thermo Scientific), anti-nanog, anti-oct4, and anti-sox2 (Gene Tex).

#### Chromatin Immunoprecipitation

Cells were plated on 100-mm tissue culture dishes and fixed with formaldehyde at a final concentration of 1% for 10-15 min. Isolated nuclei from fixed cells were sonicated using a Diagenode Bioruptor to obtain mean DNA fragmentation sizes of less than 500 base pairs. After centrifugation, lysates were pre-cleared and then incubated overnight at 4°C with 2 ug of antibody directed against REST (Millipore) or rabbit IgG, followed by protein-A/G agarose incubation for 2 hrs. After precipitation and washing, the immune complex was incubated with RNase and proteinase k at 37°C and de-crosslinked overnight at 65°C. DNA regions of interest were tested using qPCR (Openbiosystems SyBr green Mastermix).

#### Quantitative Real-Time PCR and RT-PCR

Total RNA was prepared as previously described (45). Primer sequences for the detection of calbindin, synaptotagmin4, and mash1 transcripts were obtained from a previous study (18). See Table1 for primer sequence information.

#### Alkaline Phosphatase Staining

mESC colonies were stained with Vector Blue Alkaline Phosphatase Substrate Kit I (Vector Labs), following the manufacturer's instructions. Pluripotent mESC

colonies stain positive for alkaline phosphatase (AP), whereas differentiating colonies have faint or negative AP signals. To quantify AP staining readouts, colonies were picked from three random areas and images taken using a 4X microscope objective. AP readouts were analyzed using Image J software, and the experiment was repeated three times.

#### Electrophoretic Mobility Shift Assay (EMSA)

Using a Lightshift Chemiluminescent EMSA Kit (Thermo Scientific), EMSA was performed according to the indicated instructions. The indicated recombinant proteins of REST (thrombin-cleaved from GST-REST), MBP-p120 and MBP were bacterially produced and then purified. In two different pair-wise manners, protein-protein or protein-DNA combinations were made, generating similar experimental results: either REST and MBP-p120 (or MBP alone) were incubated over-night at 4C followed by addition of the oligonucleotide; or REST was incubated with the oligonucleotide for 10 minutes, followed by addition of MBP-p120 (or MBP alone) for 10 minutes at RT. The complementary 5' biotin-labeled oligos were hybridized and encoded a single RE1 consensus sequence (10), and were used as probe :

5' biotin-

CTCTATCGATAGTTCAGCACCAAAGGACAGCGCCGGTACCGAGCTCTTA-3'

and 5' biotin-

TAAGAGCTCGGTACCGGCGCTGTCCTTTGGTGCTGAACTATCGATAGA G-3'



### Immuno-staining

Differentiating mESC colonies were fixed with 4% formaldehyde and permeabilized using 0.3% Triton X100. Anti-doublecortin (anti-DCX) antibody (Abcam) was used to detect endogenous DCX. For immuno-staining of the indicated tagged proteins expressed in pluripotent mESC colonies, anti-HA (Y-11, Santa Cruz) and anti-myc (9E10, Developmental Studies Hybridoma Bank) antibodies were used. DAPI was used for counter-staining. Images were acquired using 3i Confocal microscopy (Zeiss).

### Statistical Analysis

Using the Student's T-test within the Microsoft Excel program, and one-way ANOVA within GraphPad Prism Version 6.0a, we analyzed data significance and obtained P-values.

Table 1

qPCR primers

mGAPDH-F	TCGTCCCGTAGACAAAATGG
mGAPDH-R	TTGAGGTCAATGAAGGGGTC
mNeuro D1 -F	GAGGCTCCAGGGTTATGAGA
mNeuro D1 -R	ACTCATCTGTCCAGCTTGGG
q-mSyt4-F	AATGAGGTGATTGGACGGTTG
q-mSyt4-R	AGTGCCCCCACC GC
q-mMash1-F	TCGTCTCTCCGGA ACTG AT
q-mMash1-R	TAGCCGAAGCCGCTGAAG
mNanog-F	GGTTGAAGACTAGCAATGGTCTGA
mNanog-R	TCCAGATGCGTTCACCAGATAG
mOct4-F	TGCTGAAGCAGAAGAGGATCAC
mOct4-R	CAGATGGTGGTCTGGCTGAA
mSox2-F	AGATGCACA ACTCGGAGATCAG
mSox2-R	TCATGAGCGTCTTGGTTTTCC
mFoxA2-F	GGCACCTTGAGAAAGCAGTC
mFoxA2-R	GACATACCGACGCAGCTACA
mSox1-F	AGATGCACA ACTCGGAGATCAG
mSox1-R	GAGTACTTGTCCTTCTTGAGCAGC
miR-9-1-F	GGGTTGGTTGTTATCTTTGGTTATC
miR-9-1-R	AGACTCCACACCACTCATACAGC
miR-124a-F	CTCTGCGTGTTACAGCGG
miR-124a-R	CTCTTGGCATTACCGCGTG
miR-132-F	CTCCAGGGCAACCGTGGCTTTC
miR-132-R	TGGCTGTAGACTGTTACCTCCGGTTC
mGAD1-F	AACAAACACGGGTGCAATTT
mGAD1-R	TCACCCTCGATTTTCAACC
mSyn1-F	CCACAGGGTATGTTGTGCTG

mSyn1-R	GCCCAGATGGTTCGACTACA
mMap2-F	GCTGGTGGTATGTTCTGGCT
mMap2-R	TACCGGTTCCCTCAGCTTGTC
mDcx-F	TTCAGGACCACAAGCAATGA
mDcx-R	GGAAACCGGAGTTGTCAAAA
mBra-F	GAGCCTCGAAAGAACTGAGC
mBra-R	CAGCCCACCTACTGGCTCTA
mTubb3-F	AGTCCCCTACATAGTTGCCG
mTubb3-R	AGTCAGCATGAGGGAGATCG

#### RT-PCR primers

mMash1 RT-F	GGAAGTATGCGCTGCAAACGCCG
mMash1 RT-R	GTTGGTAAAGTCCAGCAGCTCTTGTT
mCalbindin RT-F	GTTTCGTGTATCCTTTAGCTAGTGTGT
mCalbindin RT-R	TCTAAAGTCACTGCTTCCAAATACGTGC
mSyt4 RT-F	GGTGTGCGCAAGTTTTTCATAAGATATTC
mSyt4 RT-R	GCTACCCTTCTTATGATGAGACTGTATC

#### ChIP-qPCR primers

ChIP-mSyt4-3UTR-F	CAAACAACCCCCAAAACAAC
ChIP-mSyt4-3UTR-R	CAAGGAGACACAGCCTCACA
ChIP-mCal-3UTR-F	GGGGAAACTGGGTAGATGGT
ChIP-mCal-3UTR-R	GCCTGCCTCTGTTTTCCATA
ChIP-miR124a3 5UTR-F	CCCTTTCTGGAGGAATGACA
ChIP-miR124a3 5UTR-R	ATCAACAGAAACCCGTGGAG
ChIP-miR124a3-F	ACCCCAGAGAAATGGGGTAG
ChIP-miR124a3-R	AAAGTGATCACCGCCTTCAC
ChIP-mGAPDH-F	CTGCAGTACTGTGGGGAGGT
ChIP-mGAPDH-R	CAAAGGCGGAGTTACCAGAG
ChIP-mGAD1-F	CGCACCTGCAGTGAACACC

ChIP-mGAD1-R	AAGACTTCAGCACCGAGGACA
ChIP-mSyt4-F	ACTTGCTCACCGAATTCCAC
ChIP-mSyt4-R	GAAGAGCCAACAGGAACAGG
ChIP-mCal-F	CCACCTGCTGCTTCCTAGAC
ChIP-mCal-R	CCGCACCCAGTTCTCTGTAT

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## VITA

Moonsup Lee was born in Andong, Gyung-buk, South Korea, on January 20<sup>th</sup> 1978 to Geun-Su Lee and Hu-Ja Bae. After attending Gyeong-An High School, he pursued a Bachelor's of Science degree in Biology at Korea University in Seoul, South Korea, which he earned in 2000. He achieved a Master's degree in Biochemistry at Korea University in 2002. While pursuing his Master's degree, he studied cell cycle modulation mechanisms of cancer cells upon DNA damage responses. After two years of military service in South Korea, Moonsup initiated his Doctor of Philosophy studies in Biomedical Science at The University of Texas – Houston Graduate School of Biomedical Sciences in 2008.

Permanent address:

30-3, Pyeung-hwa 9-gil, Pyeung-hwa dong, Andong, Gyung-buk, South Korea,  
760-270